Catecholamine metabolism via monoamine oxidase (MAO) within myocardium of individuals with type 2 diabetes

by

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April 2019

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The primary comorbidity and cause of mortality in type 2 diabetes is cardiovascular disease. The structural and the functional cardiac remodeling observed in patients with type 2 diabetes has been shown to be driven by a plethora of mechanisms including mitochondrial dysfunction and oxidative stress. Monoamine oxidase (MAO), an enzyme located on the outer mitochondrial membrane, catabolizes catecholamines to produce two reactive byproducts upon substrate deamination: catechol-aldehyde and H_2O_2. We hypothesized that within environments of decreased redox buffering capacity like type 2 diabetes, these byproducts of MAO metabolism disrupt mitochondrial respiration and further drive redox imbalance. Furthermore, we hypothesized that the antioxidant and aldehyde scavenging capacity of carnosine could mitigate the reactivity of MAO-derived byproducts. A comprehensive analysis of catecholamine metabolism was performed in atrial myocardium from individuals with and without type 2 diabetes. MAO-A and-B maximal activity and expression were significantly increased within myocardium from individuals with diabetes and correlated with BMI. MAO-dependent metabolism of norepinephrine decreased ATP production within myocardium from individuals with type

2 diabetes. In addition, decreased aldehyde dehydrogenase and increased basal levels of catechol-protein adducts were observed within this metabolic group. Metabolomic analysis of atrial tissue from individuals with diabetes showed decreased catecholamine levels in the myocardium, supporting an increased flux through MAOs. As a proof of concept of our second hypothesis we showed that carnosine was able to sequester DOPAL, the dopamine-derived catecholaldehyde. Carnosine also attenuated DOPAL-dependent decrease in state 3 respiration in permeabilized fibers isolated from atria. Furthermore, the production of catechol-modified proteins was mitigated by carnosine in a concentration dependent manner. In conclusion, these findings illustrate a pathogenic mechanism for MAO within type 2 diabetes, and suggest that MAO-derived byproducts, especially catecholaldehydes, contribute to redox imbalance and altered mitochondrial bioenergetics observed in myocardium of individuals with type 2 diabetes. In addition, this translational study provides a mechanistic framework for the study of carnosine or related aldehyde scavengers as therapeutic approach for the prevention of cardiac dysfunction in patients with type 2 diabetes.

Catecholamine metabolism via monoamine oxidase (MAO) within the myocardium of individuals with Type 2 Diabetes

A Dissertation

Presented to the Faculty of the Department of Pharmacology and Toxicology

East Carolina University

In Partial Fulfillment of the Requirements for the

Doctor of Philosophy Degree in Pharmacology and Toxicology

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LIST OF ABBREVIATIONS

4HNE	4-Hydroxy-nonenal
5-HT	5-Hydroxytryptamine (serotonin)
ALDH	Aldehyde Dehydrogenase
ALDH2	Aldehyde Dehydrogenase 2
AP5a	P1, P5-di(adenosine-5') pentaphosphate
ADP	Adenosine Diphosphate
ANT	Adenine Nucleotide Translocase
AR	Aldehyde/Aldose Reductase
ATP	Adenosine Triphosphate
β-PEA	Beta-phenylethylamine
BSA	Bovine Serum Albumin
CABG	Coronary Artery Bypass Graft
CAR	Carnosine
CAT	Catalase
CLG	Clorgyline
DA	Dopamine
DHBA	Dihydroxybenzylamine
DHPG	3,4-Dihydroxyphenylglycol
DOPAL	3,4-Dihydroxyphenylacetaldehyde
DOPEGAL	3,4-Dihydroxyphenylglycoaldehyde
DOPET	3,4-Dihydroxyphenylethanol
ELISA	Enzyme Linked Immunosorbent Assay
ETS	Electron Transport System
FADH2	Flavin adenine dinucleotide
G6PDH	Glucose-6-phosphate dehydrogenase
GSH	Glutathione
HF	Heart Failure

HPAA	Hydroxyphenylacetic Acid
HPAL	Hydroxyphenylacetaldehyde
IS	Internal standard
LC-MS	Liquid Chromatography Mass Spectrometry
L-Cys	L-Cysteine
LV	Left Ventricle
MAO	Monoamine Oxidase
MAOi	Monoamine Oxidase Inhibitor
MDA	Malondialdehyde
NADH	Nicotinamide adenine dinucleotide
NBT	Nitroblue Tetrazolium
NE	Norepinephrine
OxPHOS	Oxidative Phosphorylation
PC	Palmitoyl Carnitine
PCA	Principle Component Analysis
Pmfb	Permeabilized Fiber Bundle
RAA	Right Atrial Appendage
ROS	Reactive Oxygen Species
RR	Relative Risk
SDS-PAGE	Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis
SLG	Selegiline
SOD	Superoxide Dismutase
TFA	Trifluroacetic acid
TYR	Tyramine

CHAPTER 1:

INTRODUCTION

PROGRESSION OF CARDIOVASCULAR DISEASE IN TYPE 2 DIABETES

Type 2 diabetes mellitus is a progressive and complex metabolic disease characterized by hyperglycemia and insulin resistance (1-3). For individuals with type 2 diabetes the primary comorbidity and cause of mortality is cardiovascular disease (4). Sustained hyperglycemia and insulin resistance contribute to micro- and macrovascular complications within the heart, including coronary artery disease, myocardial infarction and arrhythmias (5). While each disease develops independently, they often converge at the inevitable endpoint of heart failure (HF), the risk of which is even greater in individuals with type 2 diabetes (6-9).

Alterations in cardiac function and structural remodeling, most notably left ventricular (LV) hypertrophy and diastolic dysfunction, are associated with the progression of type 2 diabetes (10-13). The broader LV remodeling is a direct reflection of cellular stress within the myocardium. Within the myocardium are cardiomyocytes which orchestrate synchronous myocardial contraction and relaxation. Adult cardiomyocytes are non-proliferative which results in cellular remodeling in areas of myocardial injury including cardiomyocyte hypertrophy and myocardial fibrosis (14). Numerous molecular mechanisms and cellular pathways contribute to myocardial damage in type 2 diabetes, most prevalent involving mitochondrial dysfunction and oxidative stress (15).

MECHANISMS CONTRIBUTING TO CARDIAC ALTERATIONS IN TYPE 2 DIABETES

Mitochondrial bioenergetics

In order to continuously support contractile functions, a high rate of ATP production occurs within the myocardium. Fatty acids are the primary substrate for ATP production and approximately 95% of the ATP in the heart is made via oxidative phosphorylation (OxPHOS) (16, 17). Through OxPHOS, ATP is made through an intricate system of redox reactions performed by complexes of the electron transport system (ETS) (18). The oxidation of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_{2)}$ supplies the system with electrons which are passed from donors to acceptors until reaching molecular oxygen. Simultaneously, protons are translocated into the inter membrane space creating potential energy in the form of a proton motive force. Protons flow down their concentration gradient into the matrix through ATP synthase and the energy produced through the dissipation of the gradient produces ATP (19). The high rate of ATP production by OxPHOS is based upon its efficiency in coupling electron flow via redox reactions to ATP production. Therefore, disruptions in OxPHOS coupling can decrease production of ATP and consequentially disrupting contractile functions.

Inefficiencies in cardiac OxPHOS have been observed in models of type 2 diabetes including decreased activity of ETS complexes and decreased phosphocreatine/ATP ratios, a proxy of mitochondrial ATP production, which suggest a mismatch in ATP supply and demand (20-23). In individuals with type 2 diabetes, the reduction in ATP production was associated with decreased contractile function (20, 22, 24). Interestingly, disruptions in cardiac OxPHOS were observed in individuals with type

2 diabetes without apparent LV dysfunction and these findings imply that dysregulation of mitochondrial metabolism precedes and contributes to structural changes (22, 24).

Oxidative stress

Oxidative stress is considered a major causal factor of cardiomyocyte damage within the diabetic condition. In addition to its role in OxPHOS, the ETS is a large source of mitochondrial reactive oxygen species (ROS) production, most notably superoxide (25). As an oxidative precursor, superoxide potentiates the production of other ROS including H_2O_2, hydroxyl radicals and reactive nitrogen species (25). The toxicity of ROS are conferred through their ability to oxidize macromolecules including unsaturated lipids, proteins/enzymes, and mitochondrial DNA (26, 27). Within the mitochondria, electrophilic radicals, most notably ROS, oxidize polyunsaturated lipids within the membranes resulting in the formation of lipid peroxidation products including 4-hydroxy-nonenal (4HNE) and malondialdehyde (MDA) (28). As reactive aldehydes, 4HNE and MDA can drive further oxidation of macromolecules including the formation of aldehyde-protein adducts (28, 29).

Evidence of ROS-induced cellular damage is prominent in myocardium of individuals with type 2 diabetes as observed by increased protein oxidation, mitochondrial and cellular DNA damage, 4HNE and MDA-aldehyde adducts and cardiomyocyte apoptosis (30-34). In addition, detoxification of ROS and reactive aldehydes are compromised in the diabetic condition. Glutathione, located in the mitochondria and cytosol, is a primary antioxidant defense system which converts H_2O_2 into water. Glutathione is maintained in the reduced state (GSH) and oxidized (GSSG) in the presence of ROS, therefore the ratio of GSH:GSSG is indicative of the cellular

redox state (35). Furthermore, superoxide dismutase (SOD) and catalase (CAT) are antioxidants responsible for the detoxification of superoxide and H_2O_2. Decreased GSH: GSSG ratio, SOD, and CAT were detected in myocardium of individuals with type 2 diabetes which indicate an underlying oxidative environment associated with diabetes (36, 37).

MONOAMINE OXIDASE

Monoamine oxidase (MAO) is an enzyme located on the outer mitochondrial membrane and metabolizes intracellular catecholamines (norepinephrine, dopamine) and biogenic amines (serotonin, tyramine). Two isoforms of MAO, MAO-A and MAO-B are ubiquitously expressed throughout the human body and share approximately 70% homology (38). Each isoform exhibits preference for substrates and inhibitors; MAO-A shows greater specificity for 5-HT (serotonin) and is inhibited by clorgyline whereas MAO-B shows greater specificity for beta-phenylethylamine (β-PEA) and is inhibited by selegiline (deprenyl) (39, 40). Both forms metabolize tyramine (TYR), dopamine (DA), and norepinephrine (NE) equally (41, 42).

Three byproducts are produced through the metabolism of catecholamines by MAO: ammonia, H_2O_2, and an aldehyde (termed ‘catecholaldehyde’), with the latter two exhibiting greater reactivity (41). The two most researched catecholaldehydes are 3,4-dihydroxyphenylacetaldehyde (DOPAL) and 3,4-dihydrophenylglycolaldehyde (DOPEGAL) which are formed from the metabolism of DA and NE, respectively (43). DOPAL and DOPEGAL are quickly metabolized by separate aldehyde detoxification pathways to form water soluble products. DOPAL is converted to an acetic acid (3,4-

dihydroxyphenylacetic acid (DOPAC)) by aldehyde dehydrogenases (ALDH) and DOPEGAL is converted to an alcohol (3, 4-dihydroxyphenylethanol (DOPET)) by aldose reductase/aldehyde reductase (AR) (44, 45). DOPAL and DOPEGAL can be metabolized by the alternate aldehyde detoxification pathway, but to a significantly lesser extent (45). Since the active sites of MAO-A and MAO-B face the inner and outer mitochondrial membranes, respectively, byproducts formed by MAO deamination enter both cytosolic and inner mitochondrial domains (46, 47).

THE PATHOGENIC ROLE OF MONOAMINE OXIDASE WITHIN CARDIOVASCULAR DISEASE

MAO has been studied extensively since the 1970's and was identified as playing a key role in the neuronal cell death associated with Parkinson's disease. However, few studies have investigated the pathogenic role of MAO within cardiovascular disease (48-51). In animal models of cardiac pressure overload, MAO-A and -B activities were shown to contribute to increased LV hypertrophy, diastolic dysfunction and cardiomyocyte death all of which are pathognomonic of HF (52-54). Additionally, similar observations were made in an animal model of type 1 diabetes wherein MAO-A inhibition attenuated diastolic dysfunction and oxidative stress within the myocardium (55). Although the mechanisms by which MAO contribute to cardiac injury within diabetes and HF are unclear, the observed oxidative damage and cellular remodeling as a result of MAO activity highlight the apparent toxic reactivity of MAO-derived byproducts.

REACTIVITY OF MAO-DERIVED H_2O_2 AND CATECHOLALDEHYDES

In vitro studies have shown MAO-derived H_2O_2 to be reactive, based upon its conversion to hydroxyl radicals, leading to the depletion of reduced glutathione, induction of oxidative damage to mitochondrial DNA, and formation of lipid peroxidation of mitochondrial membranes (56-60). Unlike H_2O_2, which requires a reductant to be converted to hydroxyl radicals, catecholaldehydes are inherently reactive due to the two functional groups within its structure, the catechol and aldehyde. Both functional groups are strong electrophiles and can bind to amino acid residues to form catecholaldehyde-protein adducts (61). DOPAL covalently modifies cysteine, lysine, and methionine residues of enzymes leading to decreased or inhibited catalytic activity (61-65). Furthermore, within neuronal cells, DOPAL and DOPEGAL generated free hydroxyl radicals which induced apoptosis through the opening of the mitochondrial permeability transition pore (66-68).

Within the 'healthy' cell, MAO-derived byproducts are quickly metabolized to stable products, yet as the cellular redox balance shifts towards an oxidative state (i.e. disease), the detoxification of catecholaldehydes becomes diminished. DOPAL, 4HNE, and MDA are substrates of and detoxified by aldehyde dehydrogenase (ALDH), yet, at concentrations which exceed physiological concentrations (10μM), 4HNE and MDA inhibited the activity of ALDH resulting in increased DOPAL concentration (69-71). ALDH activity requires the cofactor NAD+ which is synthesized from complex I of the ETS. In cell models, the inhibition of complex I by rotenone led to an increase in concentration of DOPAL due to compromised NAD+ production (72, 73). Interestingly, decreased complex I activity, decreased myocardial NAD+/NADH ratio and increased

4HNE were observed in myocardium of type 2 diabetic models suggesting disrupted catecholaldehyde detoxification (31, 74, 75).

CARNOSINE AS A REACTIVE ALDEHYDE SCAVENGER

While the inhibition of MAO is an obvious target to prevent myocardial production of catecholaldehydes the use of MAO inhibitors by individuals with diabetes is contraindicated as MAO inhibition may sustain adrenergic signaling and precipitate a hypertensive crisis. Several groups have demonstrated that the antioxidants N-acetyl cysteine, glutathione and ascorbic acid were efficacious in preventing DOPAL-protein adduct formation *in vitro*, however these compounds were ineffective in reversing or detoxifying proteins modified by DOPAL, limiting their benefits to prophylactic use (63, 72, 76).

Carnosine is an endogenously synthesized dipeptide (β-alanine and L-histidine) found in millimolar concentrations in the heart (77, 78). Carnosine is synthesized in skeletal muscle, brain and heart by carnosine synthase and metabolized in plasma by carnosinase (CN1) (79-82). The cellular activity of carnosine is multi-faceted as it exhibits pH buffering, metal chelating, antioxidant and aldehyde quenching abilities, that sugges its suitability for reducing reactivity of biogenic aldehydes (77, 83). The aldehyde quenching ability of carnosine is conferred by the histidine's imidazole group and the lysine's carboxyl group (84-87). Within proteins, the imidazole and carboxyl groups are nucleophiles and common sites of adduction by electrophilic aldehydes. The aldehydes covalently bind to the two nucleophilic groups of carnosine which serve as 'decoy' substrates, allowing carnosine to 'quench' and therefore minimize the reactivity of

aldehydes (85). *In vitro* studies have shown carnosine to covalently bind to 4HNE and MDA to form aldehyde-carnosine adducts (86, 88). Carnosine can also bind to aldehydes previously attached to proteins to create a protein-aldehyde-carnosine adduct or ‘carnosylated’ conjugate (89, 90). Aldose reductase (AR), the enzyme which metabolizes DOPEGAL, is also thought to be responsible for reducing and enhancing the water solubility of carnosine-aldehyde products (91, 92).

Carnosine improves global oxidative stress associated with obesity/insulin resistance observed by enhanced detoxification of carnosine-4HNE adducts detected in serum, urine, and liver. (93-96). Endogenous levels of carnosine in diabetic and hypertensive rats were significantly decreased compared to controls, which indicate the presence and activity of carnosine may be compromised within cardiometabolic disease (97, 98). Although the ability of carnosine to detoxify MAO-derived byproducts has not been investigated yet, these findings provide strong evidence of carnosine’s ability to attenuate oxidative stress observed within type 2 diabetes.

CENTRAL HYPOTHESIS

As observed in animal models, MAO contributes to the cardiac dysfunction associated with diabetes and HF, however the mitochondrial and cellular implications of MAO-derived byproducts within diabetes remains unknown. Therefore, the work presented in this dissertation was driven by the central hypothesis that within myocardium from individuals with type 2 diabetes, MAO byproducts contribute to dysfunction in mitochondrial bioenergetics and redox imbalance. The following aims were designed to test the central hypothesis. Our first aim was to assess MAO activity

and the consequences of MAO-derived byproducts on mitochondrial energy production within patients with type 2 diabetes and our second aim was to investigate catecholaldehyde detoxification pathways and carnosine as a potential therapeutic to scavenge catecholaldehydes. The second chapter of this dissertation explores MAO activity within human myocardium and elucidates the impact of MAO-derived byproducts on OxPHOS. The chapter also examines catechol-protein adducts and catecholaldehyde detoxification pathways in individuals with and without type 2 diabetes. Chapter three focuses on non-enzymatic detoxification of catecholaldehydes and the ability of carnosine to scavenge catecholaldehydes. The fourth chapter integrates the major findings from each chapter and proposes that the reactive byproducts produced by MAO, especially catecholaldehydes, contribute to myocardial mitochondrial dysfunction and oxidative stress observed in individuals with type 2 diabetes.

CHAPTER 2:

ENHANCED CATECHOLAMINE FLUX THROUGH MONOAMINE OXIDASE CAUSES MITOCHONDRIAL TOXICITY IN HUMAN HEART WITH DIABETES

From **Nelson MA**, Efird JT, Kew KA, Katunga LA, Beatty CN, Akhter SA, Alwair H, Robidoux J, Anderson, EJ. *In review 03/2019*.

INTRODUCTION

Following the initial reports of the Framingham Study decades ago, persistent efforts were made to uncover pathophysiological mechanisms underlying cardiac complications associated with diabetes (99). Myocardial tissue remodeling and electro-mechanical dysfunction underlie most of these complications, and the role of cardiomyocyte bioenergetics and redox imbalance in these processes has recently come to the forefront. Mitochondria are at the nexus of cardiomyocyte energetics and redox balance, which is why disruptions in energy production and mitochondrial sources of reactive oxygen species (ROS) have been explored as pathogenic factors contributing to cardiometabolic disease (100, 101). Established sources of mitochondrial ROS include NADPH oxidase and sites within the electron transport system (ETS), while lipid peroxidation of the mitochondrial membrane is a source of reactive aldehydes (e.g. 4-hydroxy-nonenal (4HNE), malondialdehyde (MDA)) (102-104). More recently, monoamine oxidase (MAO) has emerged as a significant source of both H_2O_2 and aldehydes within the myocardium (36, 105)

Two isoforms, MAO-A and MAO-B are located on the outer membrane of the mitochondrion and metabolize primary amines (e.g. norepinephrine (NE), dopamine (DA)) to produce ammonia, H_2O_2 and the highly reactive and toxic catecholaldehydes: 3,4-dihydroxyphenylglycolaldehyde (DOPEGAL) and 3,4-dihydroxyphenylacetaldehyde (DOPAL) (38, 43). Although short-lived, the toxicity of catecholaldehydes stems from their ability to form covalent aldehyde protein adducts (63, 106, 107). Catecholaldehydes are primarily detoxified via oxidation (aldehyde dehydrogenase, ALDH) or reduction (aldose reductase, AR, AKR1B1) (43, 108, 109).

A pathogenic role for MAO was described in animal models of heart failure, ischemia, and more recently, diabetic cardiomyopathy (52, 55, 57, 110). While these studies laid the groundwork for establishing MAO as a pathological factor, the translational significance of these studies and the pathophysiologic mechanisms by which MAO may be acting, remain uncertain. Therefore, we performed a comprehensive biochemical analysis of catecholamine metabolism from atrial myocardium of age-matched patients with or without type 2 diabetes, with a particular emphasis placed on MAO expression and enzymatic activity. Given that MAO-derived catecholamine metabolites (i.e., catecholaldehydes) are highly reactive, we tested the hypothesis that within myocardium from patients with diabetes, increased MAO activity contributes to mitochondrial dysfunction by disrupting oxidative phosphorylation (OxPHOS).

RESEARCH DESIGN AND METHODS

Patient enrollment, blood and atrial tissue collection. All experiments involving human subjects received approval by the Institutional Review Board of the Brody

School of Medicine at East Carolina University and the University of Iowa. Patients undergoing non-emergent elective coronary artery bypass graft (CABG) or CABG/valve surgery were enrolled between January 2011 and July 2018. Inclusion criteria included patients between the age of 50-70 without a previous history of cardiac surgery or arrhythmia. A fasted blood draw was performed before the initiation of anesthetics. Prior to placement on cardiopulmonary bypass, a portion of myocardium was biopsied in each patient after establishing a purse-string suture around the right atrial appendage. Myocardial tissue was dissected, rinsed in ice-cold PBS and used immediately for mitochondrial experiments. A separate portion of myocardium was snap frozen and maintained at -80°C for biochemical and metabolomics analysis.

Quantitative analysis of MAO-A and –B enzyme content in myocardium. Standards and samples were added to immunolon-coated 96-well assay plates and incubated overnight at 4°C. The plates were washed with PBS+0.05% Tween-20 and blocked with 5% BSA at 37°C. Samples were incubated with primary antibodies for MAO-A or –B (Abcam, Cambridge, MA) followed by incubation with horseradish peroxidase-conjugated secondary antibody. Amplex Red (Thermo Fisher Scientific, Waltham, MA) was added and the fluorescence was measured at Excitation/Emission 567/590nm.
For MAO-A and -B western blots, 20µg of sample protein lysate was loaded onto a 10% acrylamide gel and subject to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to a PVDF membrane and blocked with 5% BSA in TBS-Tween. The membranes were incubated with primary antibodies specific for each MAO isoform. The membranes were washed, probed with

secondary antibodies and imaged using Odyssey imaging system (Li-Cor Bioscience, Lincoln, NE).

MAO enzymatic activity in myocardial tissue. MAO activity was determined by measuring H_2O_2 production in the presence of Amplex Red. Each assay was measured in 1.5mLs using Amplex Red (10 µM), horseradish peroxidase (1U/mL), superoxide dismutase (25U/mL), and 15µL of myocardial lysate. Enzymatic activities were normalized to the concentration of isozyme obtained from the ELISA and reported as nmol/min/mg enzyme.

Measurement of DA and NE using LC-MS. After homogenization, samples were centrifuged at 14,000g for 30 min. The supernatant was placed under nitrogen flow, maintained at 50°C, and evaporated to dryness. The residue was resuspended in 1:1 methanol: H_2O. Standards of catecholamines (10ng/mL to 1500ng/mL) were prepared in 1:1 H_2O: methanol and maintained at -20°C prior to use. Quantitative analysis of catecholamines were performed using an Exion LC/AB SCIEX 3200 triple quadropole mass spectrometer (AB SCIEX, Framingham, MA). LC was performed using a Hypersil Gold aQ (3x50mm, 1.9µm) column (Thermo). The following gradient solvents were utilized: mobile phase A: 95:5 water:acetonitrile with 0.1% formic acid and B: 100% methanol with a flow rate of 0.3 mL/min. A 15 min total run time was used with a linear gradient from 97% B over 8 min was held for 1 min, decreased to 0% B for 1 min and equilibrated for 5 min. A 10µL injection volume was used from each sample. Parameters used for metabolite identification and quantification are outlined in Supplementary Table

1. Concentrations of DA and NE were based upon the standard curve and normalized to tissue weight.

Preparation of permeabilized muscle fibers (Pmfbs). Previous methods published from our lab were used to prepare permeabilized muscle fibers (Pmfbs) from atrial myocardium (31). After excision of myocardium from the atrial sample, ~300mg myocardium was placed in ice-cold Buffer X (7.23 mM K_2EGTA, 2.77mM CaK_2EGTA, 20mM imidazole, 0.5mM DTT, 20mM taurine, 5.7mM ATP, 14.3mM phosphocreatine, 6.56mM $MgCl_2$-$6H_2O$, 50mM KMES, pH 7.1) followed by a 30 min incubation with 3µg/mL of collagenase type I (Worthington, Lakewood, NJ) at 4°C. Using fine tip tweezers, muscle fiber bundles were separated along the longitudinal axis and permeabilized in 10µg/mL saponin for 30 min. The fibers were then incubated in the respiratory buffer containing blebbistatin until used for respiratory experiments (<1 hour). Oxygen flux was quantified using Oroboros DatLab software. To prevent extramitochondrial production of ATP, P1, P5-di(adenosine-5') pentaphosphate (AP5a), an adenylate kinase inhibitor, was added. Experiments were performed using Buffer Z-lite (10mM K-MES, 30mM KCl, 10mM KH_2PO_4, 5mM $MgCl_2$-$6H_2O$, 0.5mg/mL BSA, 20mM creatine), 1mM EGTA, 200µM $NADP^+$, 4mM glucose, 1.7U/mL of glucose-6-phosphate, and 3.4U/mL of hexokinase. The recordings were normalized for 10 min followed by the addition of 50µM palmitoyl-L-carnitine and 2mM malate. After 3 min, 500µM ADP was added to drive maximal respiration. After each experiment myofibers were collected and stored at -20°C in a freezing buffer (Buffer X, glycerol, protease inhibitor cocktail).

Mitochondrial analysis in permeabilized human cardiac myofibers. Simultaneous and real-time ATP/O_2 measurements were measured using a customized system which connected a spectrofluorometer (HORIBA Scientific, Edison, NJ) to an Oroboros O2K Oxygraph (Oroboros, Innsbruck, Austria) via lightguides. The glucose-6-phosphate dehydrogenase/hexokinase coupled enzyme system links the conversion of glucose, via mitochondrial ATP production, to the reduction of $NADP^+$ to NADPH which is autofluorescent at Ex/Em 345/460. The rate of ATP production was calculated based on a standard curve of fluorescence corresponding to known ATP concentrations. Oxygen flux was quantified using Oroboros DatLab software. Experimental conditions are as outlined in the Figure legends.

Normalization of mitochondrial content in permeabilized muscle fibers. To estimate mitochondrial content within Pmfbs, citrate synthase activity was measured based upon published methods (111). Fibers were removed from freezing buffer, homogenized 1:20 (w/v) in Tris- buffer (100mM Tris-base, 2mM EDTA pH 7.5) and centrifuged for 10 min at 10,000g at 4°C. Homogenates were diluted 1:10 with Tris-buffer and added onto a clear polysterene 96 well plate. A reaction cocktail including 5,5'-Dithiobis(2-nitrobenzoic acid), acetyl-CoA, and oxaloacetate was added to each well and absorbance was measured at 412nm every min for 10 min. Citrate synthase activity was calculated using the extinction coefficient of 13.6 L $mol^{-1}cm^{-1}$ and the results were used to normalize respiratory experiments.

Detection of catecholaldehyde-modified proteins Nitroblue tetrazolium (NBT) is a redox-cycling dye which binds to catechol-protein adducts within a nitrocellulose membrane and stains the proteins blue, allowing the visualization of catechol-modified proteins (70, 112). Myocardial lysate alone, or after having been incubated with catecholamines -/+ MAO inhibitors, was subjected to SDS-PAGE. The gel, filter paper, and nitrocellulose membrane were incubated in Bjerrum Schaefer-Nielsen transfer buffer (48mM Tris-Base, 39mM glycine, 20% methanol) and transferred at 10V for 70 min. The membrane was incubated in NBT solution (0.24mM NBT in 2M potassium glycine buffer) in the dark for 40 min followed by imaging using a ChemiDoc Imaging System (Bio-Rad, Hercules, CA).

ALDH2 activity and protein expression. Aldehyde dehydrogenase 2 (ALDH2) is the mitochondrial isoform of aldehyde dehydrogenase (ALDH) and is responsible for metabolizing catecholaldehydes into weak acids. ALDH2 activity was measured based upon previous methods and determined by monitoring the ALDH2 conversion of NAD^+ to NADH reflected by an increase in absorbance at 450nm (113). Assays were performed using 50mM sodium pyrophosphate buffer (pH 8.0), 200µg of cardiac protein lysate, 2.5mM NAD+, and 2µM rotenone. The reaction was initiated with the addition of 10mM acetaldehyde and absorbance was recorded every 30s for 15 min. The detection of ALDH2 within myocardial lysates was performed using Western Blot techniques previously noted using a primary antibody targeting ALDH2 (Abcam).

Metabolomic profiling of myocardial samples. An Eksigent 425 microLC/SCIEX 5600+ time-of-flight mass spectrometer (AB SCIEX) was used to measure metabolites within myocardial samples. Samples were prepared the same as the NE and DA LC-MS preparation. A Halo C18 0.5 x 50 mm 2.7 µm column used for separation of the metabolites with the following solvent compositions: mobile phase A: 95:5 water:acetonitrile with 0.1% formic acid and B: acetonitrile with 0.1% formic acid. A linear gradient was utilized with a flow rate of 10 µL/min where the gradient started with 10% B for 2 min, increased to 90% B for 15 min, held for 5 min, dropped to 10% B over 2 min and equilibrated for 10 min for a total run time of 30 min. Injection volume for samples was 5 µL. Data was acquired for MS and MS/MS analysis using independent data acquisition for the top 20 ions in positive ionization mode. The scan range for MS was 80-1250 Da. Principal component analysis (PCA) and t-tests were conducted using MarkerView 1.3.1. Statistically significant masses were tentatively identified using the Human Metabolome Database (hmbd.ca) and confirmed with MS-MS or a standard. Pathway analysis was then conducted via Metaboanalyst (http://www.metaboanalyst.ca) on database identified peaks (114, 115).

Statistical Analysis. In the human clinical data analysis, categorical variables are presented as frequency and percentage and continuous variables were presented as mean ± standard deviation. Fisher exact and Chi (χ^2) procedures were used to compute statistical significance of group comparisons for categorical variables. The Deuchler-Wilcoxon test was used for continuous variables. For the multivariable analysis, quartiles of MAO-A and –B activity were analyzed using a robust Poisson regression

model with relative risk (RR) of diabetes as the measure of association. Missing values for all clinical and biochemical variables (<5%) were imputed using the iterative expectation-maximization (EM) algorithm (30, 36). *P*-value in multivariable analysis was computed using Friedman's nonparametric test for central tendency while adjusting for age, sex and race. P_{trend} was computed using likelihood ratio trend test, adjusting for age and sex. SAS Version 9.4 (Cary, NC) was used for all analyses of human biochemical and clinical variables. For MAO kinetic measurements, best-fit curves were implemented using nonlinear regression analysis and rates of MAO activity were compared between metabolic groups at each titration timepoint.

The LC-MS, ELISA, ALDH2 activity and immunoblot densitometry were analyzed using unpaired Students t-tests to compare values between metabolic groups. Multiple paired Student's t-test were used to compare differences in treatment groups of mitochondrial respiration experiments. Metabolomic data was analyzed using PCA followed by Student's t-test to compare peak values between metabolic groups. Data was presented as mean ± SEM and values of $p<0.05$ were considered statistically significant. All analysis was performed using GraphPad Prism 8 (GraphPad Software, La Jolla, California).

RESULTS

MAO expression and activity is up-regulated in right atria of patients with diabetes. For this study, samples of right atrial myocardium were analyzed from 96 patients, 51 with type 2 diabetes and 45 with normal glycemic control. As detailed in Table 1, the patients from each group did not differ in mean age or mean BMI. No

significant differences in pre-operative medications were observed between groups, with exception of the angiotensin-converting enzyme inhibitors/angiotensin II receptor blockers and the antihyperglycemics, as would be expected (Table 1).

Since MAO metabolizes intracellular catecholamines, we first measured catecholamine levels within the myocardium. NE was found to be the predominant catecholamine within the samples, and patients without diabetes showed significantly greater concentrations of DA ($p<0.001$) and NE ($p=0.012$) in their myocardium, as compared to patients with diabetes (Figure 1A). It was recently shown that MAO expression is increased in heart with insulin resistance/diabetes (55, 116). Therefore, we measured myocardial MAO enzyme content and activity in myocardial extracts. Results from the quantitative ELISA revealed that the predominant MAO isoform within atrial myocardium was MAO-A. In univariable analysis, both MAO isoforms were significantly increased within myocardium from patients with diabetes ($p<0.05$) at total enzyme level (Figure 1B and 1C). After adjusting for age, sex, and race, multivariable analysis revealed that only MAO-A was significantly higher in patients with diabetes ($p=0.024$) (Table 2).

To further characterize MAO, kinetic assays were performed using clorgyline (MAO-A inhibitor) and selegiline (MAO-B inhibitor) to distinguish isoform specific activity. Assay specificity for each isozyme is shown with these inhibitors in Supplemental Figure 1. MAO kinetics differed between metabolic groups and the effect was variable across the monoamine substrates tested. The observed V_{max} for both MAO-A and –B isoforms were higher in patients with type 2 diabetes when DA was used as the substrate. MAO-A, but not -B, displayed a higher observed V_{max} when NE

was used (Figure 1D). Multivariable analysis confirmed that MAO-A activity supported by DA and NE was significantly increased within myocardium from patients with diabetes (Supplemental Table 2.1 and 2.2). With β-phenylethylamine (βPEA), the observed V_{max} of MAO-A and –B appeared to be lower in patients with diabetes (Figure 1D). No significant effect was observed between metabolic groups when 5-HT or TYR were used as substrates. Taken together, these results indicate that MAO expression is increased, while enzymatic activities are altered in a monoamine substrate-specific manner in diabetes.

Irrespective of metabolic status, over 70% of the patients within the cohort were determined to be either overweight or obese (BMI 25-33.3). Therefore, we sought to determine if there was an association between MAO and obesity and determined that BMI is associated with dopamine-supported maximal activity of both isoforms MAO-A (R^2=0.23, p=0.001) and MAO-B (R^2=0.21, p=0.002) (Figure 2A and B). An association was also found between glycated hemoglobin (HbA1c) levels and MAO-A (R^2=0.12, p=0.03) and MAO-B (R^2=0.16, p=0.01, Fig. 2C and D).

Table 1. Patient characteristics and preoperative medications (n=96).

Variables	Diabetes n (%)	Non-Diabetes n(%)	p-value
Age			
Mean±SD	66±9.2	67±9.9	0.6093[+]
BMI			
Mean±SD	30±7.1	29±6.2	0.4669[+]
Race			
White	36 (71)	39 (87)	0.083[++]
Black	15 (29)	6 (13)	
Sex			
Female	9 (18)	5 (11)	0.40[++]
Male	42 (82)	40 (89)	
Preoperative Drugs			
ACEIs/ARBs	32 (63)	14 (31)	0.0023[++]
Antihyperglycemics	37 (73)	2 (4)	0.0001[++]
Beta-Blockers	39 (76)	31 (69)	0.49[++]
Calcium Channel Blockers	11 (22)	8 (18)	0.80[++]
Diuretics	19 (37)	15 (33)	0.83[++]
Nitrates	30 (59)	25 (56)	0.84[++]
Statins	40 (78)	34 (76)	0.81[++]

* p-value computed using Student's t-test
** p-value computed using Fisher's exact test

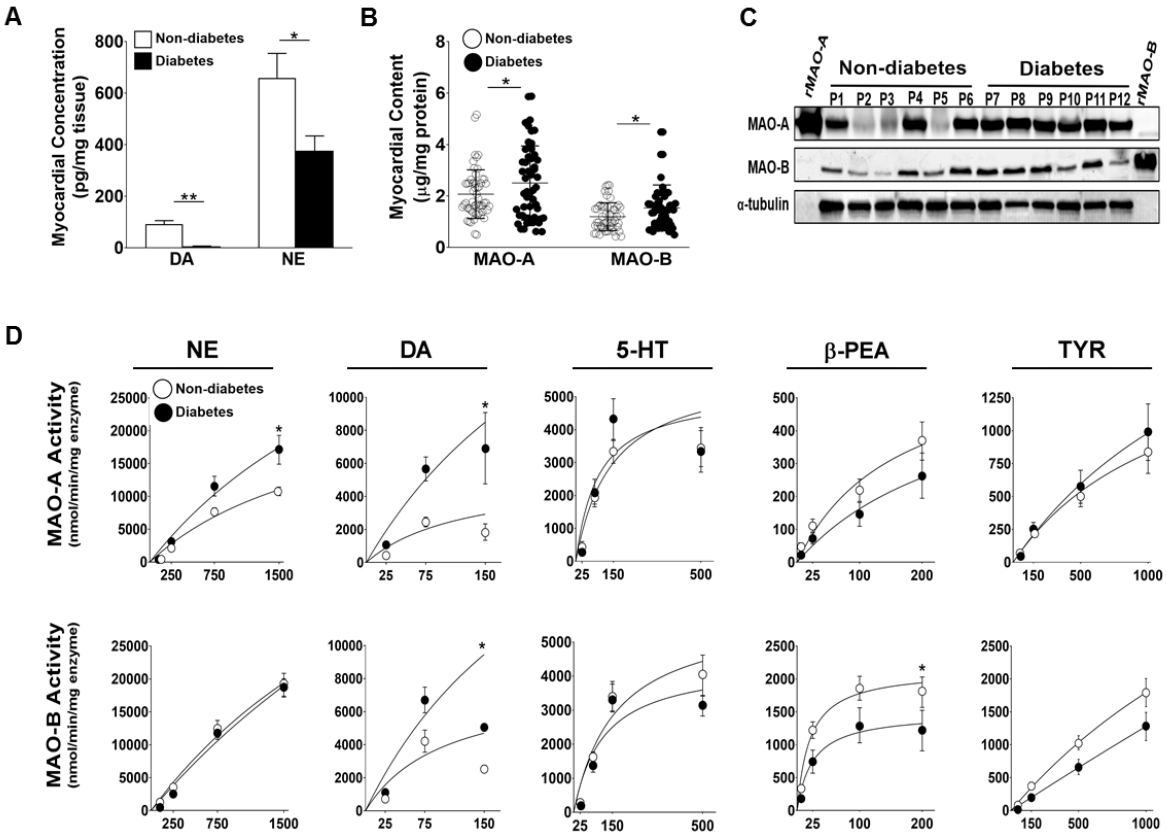

A
Myocardial Concentration (pg/mg tissue)
Non-diabetes
Diabetes
DA
NE
B
Myocardial Content (μg/mg protein)
MAO-A
MAO-B
C
rMAO-A
rMAO-B
Non-diabetes
Diabetes
P1 P2 P3 P4 P5 P6 P7 P8 P9 P10 P11 P12
MAO-A
MAO-B
α-tubulin
D
NE
DA
5-HT
β-PEA
TYR
MAO-A Activity (nmol/min/mg enzyme)
MAO-B Activity (nmol/min/mg enzyme)

Figure 1. Cardiac MAO protein expression and enzymatic activity in human myocardium

LC-MS analysis of dopamine and norepinephrine concentrations in human atrial myocardium (A) from patients with type 2 diabetes and non-diabetes (n=15/metabolic group). MAO-A and -B concentrations measured using MAO isoform specific antibodies via (B) ELISA (n=45-55/metabolic group) and (C) western blot, α-tubulin was used as the loading control (n=6/metabolic group). Kinetic traces of MAO activity (D) using 5 biogenic substrates: norepinephrine, dopamine, serotonin, beta-phenylethylamine and tyramine. MAO activity was measured by monitoring the increase in fluorescence of resorufin at Ex/Em: 571/585. MAO-A activity is shown on the top panel and MAO-B activity is on the bottom panel (n=20/metabolic group). Data are represented as ±SEM. *p<0.05, **p<0.005 compared with non-diabetes. 5-HT, serotonin. βPEA, beta-phenylethylamine. DA, dopamine. NE, norepinephrine. TYR, tyramine.

Table 2. Multivariable analysis for MAO content and diabetes status.

MAO-A Total (µg/mg)			
	Diabetes	Non-Diabetes	P-value
Mean±SD	2.7±1.3	2.2±0.97	0.018†
			P_{trend} =0.024††
MAO-B Total (µg/mg)			
Mean±SD	1.3±0.76	1.1±0.51	0.47†
			P_{trend}=0.24††

† p-value computed using Friedman's Nonparametric test for central tendency, adjusting for age, sex and race.

†† p-value computed using the likelihood ratio trend test, adjusting for age, sex and race.

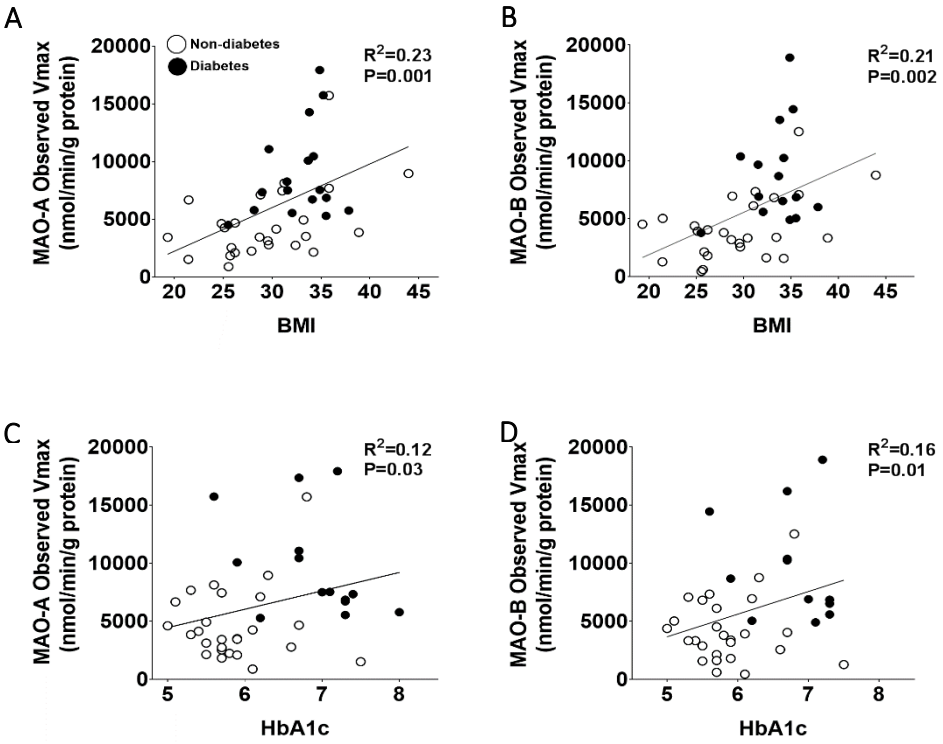

A
Non-diabetes
Diabetes
R^2=0.23
P=0.001
MAO-A Observed Vmax (nmol/min/g protein)
BMI
B
R^2=0.21
P=0.002
MAO-B Observed Vmax (nmol/min/g protein)
BMI
C
R^2=0.12
P=0.03
MAO-A Observed Vmax (nmol/min/g protein)
HbA1c
D
R^2=0.16
P=0.01
MAO-B Observed Vmax (nmol/min/g protein)
HbA1c

Figure 2. Maximal MAO activity and association with metabolic parameters

Shown are pairwise correlations between BMI and observed Vmax of (A) MAO-A and (B) MAO-B. In (C) and (D) are pairwise correlations between HbA1c and observed Vmax of MAO-A and MAO-B, respectively. For this analysis MAO activity was measured using dopamine. n=26 for non-diabetes and n=16 for diabetes.

MAO activity disrupts mitochondrial OxPHOS in myocardium patients with diabetes. Metabolism of TYR and DA by MAO caused suppression of mitochondrial respiration in isolated rat brain mitochondria, but whether catecholamine metabolism by MAO disrupts mitochondrial bioenergetics within the heart is not known (117, 118). To address this question, we measured mitochondrial OxPHOS (ATP production/O_2 consumption) using permeabilized myofibers (Pmfbs) prepared from fresh samples of atrial myocardium. In Pmfbs isolated from patients without type 2 diabetes, incubation with NE did not significantly alter ATP production or O_2 consumption (Figure 3A and B). Metabolism of NE by MAO significantly decreased ATP production ($p<0.05$) in Pmfbs from patients with type 2 diabetes while O_2 consumption was not significantly affected (Figure 3A and B). This attenuation in ATP production corresponded to a decrease in ATP/O ratio (Figure 3C). This effect was confirmed to be driven by MAO metabolism of NE, as concurrent treatment with MAO inhibitors attenuated the effect.

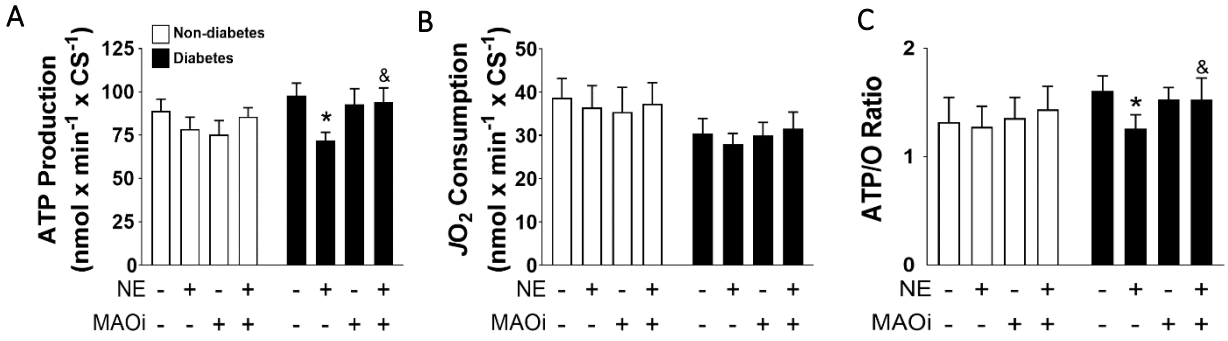

A
Non-diabetes
Diabetes
ATP Production
(nmol x min-1 x CS-1)
125
100
75
50
25
0
*
&
NE - + - + - + - +
MAOi - - + + - - + +
B
JO2 Consumption
(nmol x min-1 x CS-1)
50
40
30
20
10
0
NE - + - + - + - +
MAOi - - + + - - + +
C
ATP/O Ratio
2
1
0
*
&
NE - + - + - + - +
MAOi - - + + - - + +

Figure 3: MAO-derived byproducts disrupt OxPHOS. Simultaneous (A) ATP production, (B) O_2 consumption and (C) ATP/O ratio were measured in permeabilized fibers prepared from atrial myocardium treated with: no MAO substrate or inhibitors, 75µM NE, MAO inhibitors, or MAO inhibitors + 75µM NE. n=10/metabolic group with 4 fibers from each patient. *p<0.05 compared to control, &p<0.05 compared to NE.

Metabolism of catecholamines by MAO generates catechol-protein adducts in extracts of atrial myocardium. DOPAL and DOPEGAL are quickly metabolized by ALDH2 and AR into acetic acid (dihydroxyphenylacetic acid, DOPAC) and alcohol (dihydroxyphenylglycol, DHPG) respectively, which are their primary routes of metabolism (Figure 4A). However, when these detoxification pathways are overwhelmed, catecholaldehydes have been shown to form catechol-protein adducts (43, 49, 63, 76). To our knowledge, there has been no previous study examining formation of catecholaldehydes or catechol-modified protein adducts in myocardial tissue. Therefore, we first measured unbound DOPAL and DOPAC in plasma and myocardial extracts using quantitative MS/MS approach. In plasma, free DOPAL level were increased in patients with type 2 diabetes, while DOPAC levels were found to be similar between the metabolic groups (Supplemental Figure 2). Within myocardium, DOPAL and DOPAC concentrations were below the detectable limit of the instruments used.

This was not surprising, however, because owing to their high reactivity, free catecholaldehydes are unlikely to be present in myocardium. Therefore, we refined a technique using nitroblue tetrazolium (NBT), to determine if myocardial tissue contains catechol-modified protein adducts. NBT is a dye that selectively reacts with the catechol moiety on proteins to form a stable blue conjugate on nitrocellulose membrane (Figure 4B). Myocardial extracts all contain catechol-modified protein adducts at baseline, with substantially greater levels within myocardium from patients with diabetes (Figure 4C). Extracts exposed to NE, and to a lesser extent, DA, revealed an increase in catechol-modified protein adducts (Figure 4D). The catechol modifications were determined to be

dependent upon MAO activity, which strongly suggests that MAO-derived catecholaldehydes are responsible for this modification (Figure 4E).

Within the mitochondria, aldehydes formed from lipid peroxidation (4HNE, MDA) are metabolized by ALDH2, the same enzyme which primarily metabolizes DOPAL, and to a lesser extent, DOPEGAL (30, 70, 106). Studies have shown that 4HNE and DOPAL, at high concentrations, can allosterically inhibit ALDH2 activity, thereby creating a ‘bottleneck’ for aldehyde detoxification (69, 71). Furthermore, a recent study from our lab revealed that myocardium from patients with type 2 diabetes contain greater amounts of 4HNE compared to patients without diabetes, suggesting basal aldehyde stress within the cardiac mitochondrial microenvironment of these patients (30). In animal models of diabetes, the attenuation of ALDH2 activity lead to an increase in aldehyde load and contributed to left ventricular dysfunction (119, 120). Since ALDH2 is involved in catecholaldehyde metabolism, ALDH2 content and activity was measured in atrial myocardium. Interestingly, while there were no differences in ALDH2 enzyme content between the metabolic groups (Figure 4F and G), ALDH2 activity was significantly reduced ($P<0.039$) in patients with type 2 diabetes (Figure 4H).

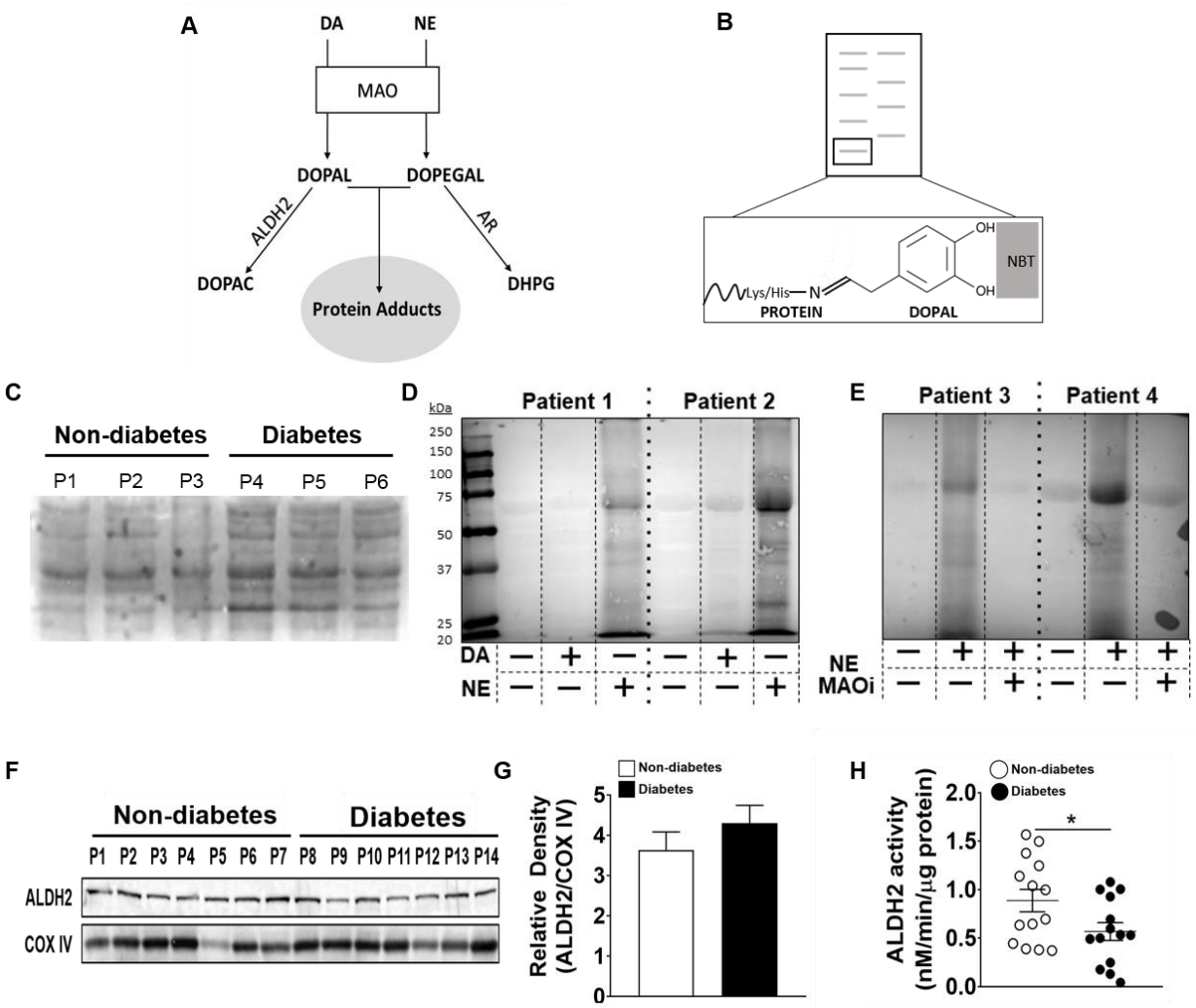

A
DA
NE
MAO
DOPAL
DOPEGAL
ALDH2
AR
DOPAC
Protein Adducts
DHPG
B
OH
NBT
Lys/His
N
OH
PROTEIN
DOPAL
C
Non-diabetes
Diabetes
P1
P2
P3
P4
P5
P6
D
kDa
Patient 1
Patient 2
250
150
100
75
50
37
25
20
DA
NE
E
Patient 3
Patient 4
NE
MAOi
F
Non-diabetes
Diabetes
P1 P2 P3 P4 P5 P6 P7 P8 P9 P10 P11 P12 P13 P14
ALDH2
COX IV
G
Non-diabetes
Diabetes
Relative Density
(ALDH2/COX IV)
5
4
3
2
1
0
H
Non-diabetes
Diabetes
ALDH2 activity
(nM/min/μg protein)
2.0
1.5
1.0
0.5
0.0
*

Figure 4: Detection and metabolism of catecholaldehydes in myocardium Pathway of catecholamine metabolism into catecholaldehydes (by MAO) and then into acetic acid (DOPAC) or alcohol (DHPG) by ALDH2 or AR, respectively (A). If metabolism by ALDH2 or AR is compromised, catecholaldehydes can form carbonyl adducts with proteins. Shown in (B) is a representative image of catechol-protein adduct detection by NBT staining. In the presence of catechol-modified proteins NBT stains proteins blue on nitrocellulose membrane. Representative NBT stains of cardiac lysate untreated(C), after overnight incubation with DA (25μM) or NE (75μM) (D), and after overnight incubation with MAO inhibitors +/- NE (75μM) (E). Western blot analysis (F) and densitometric analysis (G) of ALDH2 in myocardium. COX IV was used as a loading control to calculate relative density. n=7/metabolic group. ALDH2 activity (H) measured by monitoring the increase in absorbance of NADH at 450nm in myocardial samples. n=14/metabolic group. *$p<0.05$. All data are shown as mean±SEM. ALDH2, aldehyde dehydrogenase 2. AR, aldose reductase. COX IV, complex IV. DA, dopamine. DHPG, dihydroxyphenylglycol. DOPAC, dihydroxyphenylacetic acid. MAOi, Monoamine oxidase inhibitors. NE, norepinephrine. NBT, nitroblue tetrazolium.

Metabolomic profiling shows pathways of catecholamine synthesis and metabolism are altered in heart with diabetes. To examine whether the changes in MAO and ALDH2 corresponded to alterations in total catecholamine flux within the myocardium, global metabolomics analysis was performed on atrial tissue from a subset of patients in each group. LC/MS peak profiling revealed 592 unique metabolite features across all samples. Principle component analysis (PCA) results showed defined clustering by diabetes status (Fig. 5A). After analysis of the 592 metabolites, 110 features were found to be statistically different between metabolic groups (Fig. 5B). Based upon m/z and retention times, the metabolites were queried and identified using the human metabolomic database (HMDB) and compiled into MetaboAnalyst™ for pathway analysis. Results showed that the three most significant pathways that were significantly different were phenylalanine, tyrosine and tryptophan metabolism pathways (Fig. 5C). The tyrosine metabolism pathway is broad and responsible for the synthesis and metabolism of TYR, DA and NE and includes associated MAO and ALDH byproducts. Based upon further MS/MS analysis, we identified and confirmed 5 metabolites within the tyrosine/catecholamine pathway that were significantly different in the myocardial tissue from the diabetes patients (Supplemental Table 3).

Tyrosine can either be converted to TYR or L-DOPA, the product of tyrosine hydroxylase. TYR is then converted to its associated catecholaldehyde, hydroxyphenylacetaldehyde (HPAL), by MAO. Samples from patients with diabetes had much lower levels of hydroxyphenylacetic acid (HPAA) ($p<0.05$), the metabolite of HPAL produced by ALDH, which is consistent with reduced ALDH activity in these patients. L-DOPA, the precursor of DA and NE synthesis ($p<0.05$), and DA ($p<0.005$)

and NE ($p<0.05$), were also decreased with diabetes. While no differences were observed within DA metabolism, dihydroxyphenylglycol (DHPG), the alcohol metabolite of DOPEGAL, was substantially decreased in myocardial tissue from diabetes patients ($p<0.05$), (Fig. 5D).

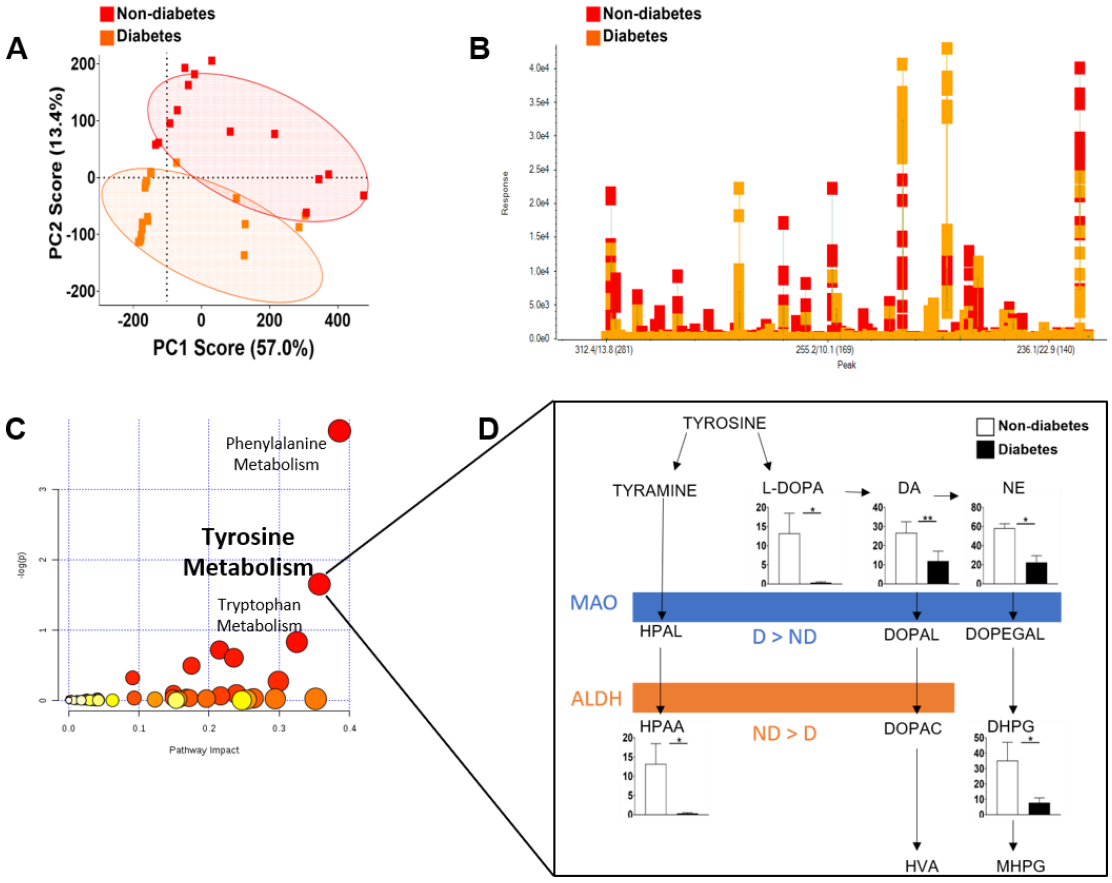

A
Non-diabetes
Diabetes
PC2 Score (13.4%)
PC1 Score (57.0%)
B
Non-diabetes
Diabetes
Response
Peak
C
Phenylalanine Metabolism
Tyrosine Metabolism
Tryptophan Metabolism
-log(p)
Pathway Impact
D
TYROSINE
TYRAMINE
L-DOPA
DA
NE
MAO
HPAL
D > ND
DOPAL
DOPEGAL
ALDH
HPAA
ND > D
DOPAC
DHPG
HVA
MHPG
Non-diabetes
Diabetes

Figure 5: Metabolomics pathway analysis of human atrial myocardium. LC-MS analysis of metabolic profiles was performed using cardiac lysate. (A) is a representative PCA scores plot showing clusters based on metabolic status. (B) After alignment and data extraction 592 metabolite features were detected of which 110 were found to be statistically significant between metabolic groups ($p \leq 0.05$). (C) Metaboanalyst pathway analysis revealed that five of the metabolites were involved within the tyrosine metabolism pathway (D). The five metabolites shown in the abbreviated tyrosine pathway were confirmed by comparison of the observed mass fragmentation patterns and retention times to those published in the Human Metabolome Database. Peak areas of the identified metabolites are compared and shown in D. All data are shown as mean±SEM. *$p<0.05$, **$p<0.005$ compared with non-diabetes. DA, dopamine. DHPG, dihydroxyphenylglycol DOPAC, dihydroxyphenylacetic acid. DOPAL, dihydroxyphenylacetaldehyde. DOPEGAL, dihydroxyphenylglycolaldehyde.NE, norepinephrine. HPAA, hydroxphenylacetic acid. HPAL, hydroxyphenylacetaldehyde. HVA, homovanillic acid. MHPG, 3-methoxy-4-hydroxyphenylglycol.

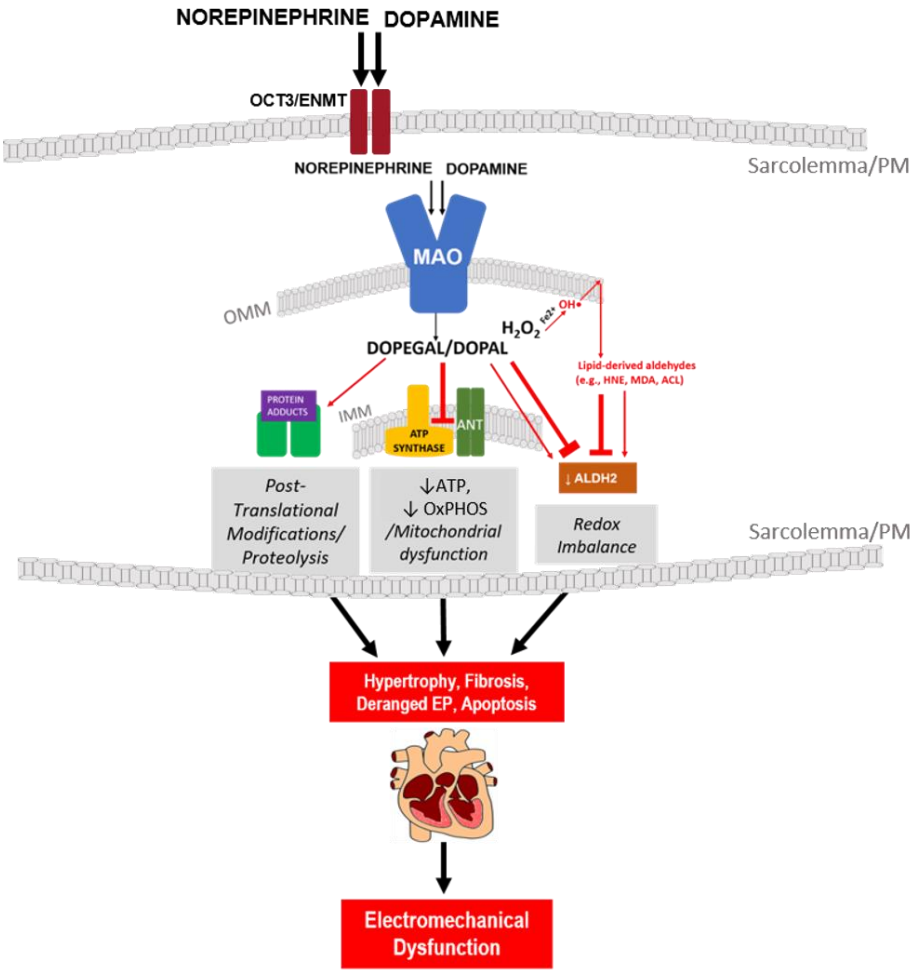

NOREPINEPHRINE DOPAMINE
OCT3/ENMT
NOREPINEPHRINE DOPAMINE
Sarcolemma/PM
MAO
OMM
H_2O_2
$Fe2^+$
$OH^•$
DOPEGAL/DOPAL
Lipid-derived aldehydes
(e.g., HNE, MDA, ACL)
PROTEIN ADDUCTS
IMM
ATP SYNTHASE
ANT
↓ ALDH2
Post-Translational Modifications/ Proteolysis
↓ATP, ↓ OxPHOS /Mitochondrial dysfunction
Redox Imbalance
Sarcolemma/PM
Hypertrophy, Fibrosis, Deranged EP, Apoptosis
Electromechanical Dysfunction

Figure 6: Schematic of MAO-derived byproducts within myocardium from patients with type 2 diabetes. MAO expression and activity are increased in myocardium of patients with type 2 diabetes, suggesting increased flux of catecholamines through MAO. In myocardium, MAO-derived byproducts form catechol-modified protein adducts (post-translational modifications) and decrease ATP production (energy disruption). In diabetes patients, the compromised ALDH2 activity would exacerbate these toxicities by creating a 'detoxification bottleneck'). Long term toxicities and carbonyl stress due to MAO-derived byproducts could contribute to hypertrophy, fibrosis, and electrophysiological (EP) derangements, ultimately leading to electromechanical dysfunction in myocardium of diabetes patients. OCT3/ENMT = organic cation transporter-3/extraneuronal monoamine transporter; OMM = outer mitochondrial membrane; IMM = inner-mitochondrial membrane; ANT = adenine nucleotide translocase; OxPHOS = oxidative phosphorylation; EP = electrophysiology

DISCUSSION

This translational study provides direct evidence that there is a shift in catecholamine metabolic pathways within the myocardium of patients with type 2 diabetes. These findings suggest that within the myocardium, MAO activity increases, while ALDH activity decreases, and this has potentially profound impacts on redox balance and OxPHOS. Global metabolomics confirmed our biochemical analyses and suggest that increased MAO and decreased ALDH activities in myocardium from patients with diabetes are shunting catcholaldehydes (DOPAL and DOPEGAL) into alternative metabolic pathways, including formation of toxic protein adducts.

Myocardium from patients with type 2 diabetes showed a substantial decrease in DA and NE concentration, compared to patients without diabetes. We propose that the increased MAO-A and MAO-B expression and activity in these patients' myocardium is responsible for this, as enhanced MAO activity would increase catecholamine flux. In a previous study of human myocardium, MAO expression was measured in atrial samples from patients who had heart failure (HF), or HF and diabetes combined, and no significant differences in MAO-A or -B expression were found between groups (121). However, while that group used real-time PCR and immunohistochemistry in fixed myocardial tissue, we performed immunoblot and ELISA analysis after validating the specificity of the MAO-A and -B antibodies using immunoblot with recombinant forms of each enzyme (Figure 1C). We then developed a quantitative ELISA method with these antibodies to determine isoform concentrations within a large cohort of patients and combined that with a comprehensive kinetic analysis of MAO-A and –B activity in the tissue. Therefore, we are quite confident in our results showing the increase of MAO-A

and MAO-B within patients with type 2 diabetes. A more interesting and potentially important finding in the MAO analysis is that both MAO-A and –B are catalytically altered in patients with diabetes. By normalizing the enzymatic activity to isoform content (via ELISA), we were able to determine that there are altered kinetics at the level of the enzyme itself. To our knowledge, this is the first time that the kinetics of these critical enzymes have been assessed in human myocardium. These findings also suggest that MAO catalytic activity is regulated at the post-translational level (e.g., phosphorylation, acetylation, carbonylation, etc), and that some post-translational modification of MAO belies the increased catalytic activity in the myocardium with diabetes.

We found that metabolism of NE by MAO decreased mitochondrial ATP production in Pmfbs isolated from myocardium from patients with type 2 diabetes. This decrease in ATP production without a simultaneous change in oxygen consumption implies a decreased efficiency of the phosphorylation system (e.g., ATP-synthase, adenine nucleotide translocase, ANT), as opposed to a disruption in electron flux within the ETS. Since the effect of NE was mitigated by MAO inhibitors, it is most likely due to the reactivity of MAO-derived byproducts. Both H_2O_2 and aldehyde-dependent post-translation modifications of the ATP synthase have been reported to affect ATP synthase efficiency (122-124). Future studies are warranted to elucidate mechanisms by which MAO-derived byproducts disrupt ATP production, and the extent to which these byproducts contribute to cardiomyocyte electromechanical dysfunction (Figure 6).

While the potential pathogenic role of catecholaldehydes within the heart is not known, catecholaldehydes have been studied in models of neurodegenerative disease,

specifically in the pathogenesis of Parkinson's disease. DOPAL induces protein aggregation, cell death, and inhibit tyrosine hydroxylase, the enzyme responsible for DA and NE biosynthesis (62, 107, 125, 126). Furthermore, these studies established that DOPAL and DOPEGAL induce mitochondrial-mediated cell death via opening of the permeability transition pore in dopaminergic cell models (66, 67, 117). Taking into consideration that subunits of the ATP-synthase comprise the permeability transition pore, it could be speculated that the decrease in ATP production observed in Pmfbs exposed to NE was due to transient opening of the PTP induced by catecholaldehydes.

Our study also revealed ALDH2 activity to be compromised within myocardium from patients with type 2 diabetes. This mitochondrial-localized enzyme has a critical role in phase 2 detoxification and metabolism in oxidative tissues. Numerous studies of cardiac disease, including experimental models of diabetes, demonstrated the importance of ALDH2 in preserving myocardial structure and function (71, 127). Furthermore, therapeutic strategies that increase ALDH2 activity (Alda-1) or sequester aldehydes have shown promise in diabetes-associated cardiac complications (119, 128). ALDH2 is among many enzymes responsible for metabolism of DOPAL and DOPEGAL. Although DOPAL or DOPEGAL within the myocardium were below the detectable limits of our MS instruments, we showed that catecholaldehyde protein adducts are present in human myocardium, and MAO contributes to their production. Most importantly, we found that catechol-modified protein adducts are more abundant in myocardial tissue from diabetes patients (Figure 4C). Taken together, these findings strongly suggest that an aldehyde 'detoxification bottleneck' may be present in the heart with diabetes, and that catecholaldehydes are diverted more toward catechol-

modification of proteins, thereby contributing to cardiotoxicity via carbonyl stress and redox imbalance in the myocardium of these patients.

Among our patient cohort, there is variability in duration of time by which patients were treated with various medications for diabetes and/or cardiovascular disease. This was not accounted for in the present study as this data is not routinely captured in clinical records, thus we cannot exclude the possibility that duration of drug treatment (e.g. β-blockers, CCB's, anti-hyperglycemics) may have somehow affected MAO isoform content and/or activity.

Additionally, since the highest degree of sympathetic innervation in the heart is localized to the atrium, we cannot exclude the possibility that some MAO content/activity could be coming from post-ganglionic nerve terminals. Never-the-less, the fact that MAO is altered at both activity and enzyme kinetic level and that in Pmfbs, which are void of non-myocardial tissue, there is a significant effect of NE on mitochondrial ATP production in patients with diabetes, leads us to conclude that there is indeed a localized effect of MAO in myofibers that impacts mitochondrial OxPHOS.

Finally, we were not able to directly quantify total amounts of catecholaldehydes (DOPAL, DOPEGAL) in myocardial extracts and only captured adducts formed on proteins via NBT staining. There are several reasons for this. First, catecholaldehydes in tissue are highly reactive and they spontaneously and rapidly react with solvent-accessible Lys and Cys residues on proteins or are further metabolized via phase 2 enzymes (48, 61). Additionally, these adducts are proving to be very challenging to measure with MS, due to their instability and reactivity. For example, DOPEGAL cannot be easily synthesized easily is capable of self-polymerization and rearrangement, thus it

is difficult to quantify. Despite these challenges we are confident that with continued refinement in our LC/MS and MS/MS methods we will have a highly sensitive and reproducible detection method in place very soon.

This study provides direct evidence that catecholamine flux is significantly enhanced in human myocardium with diabetes, and that MAO-derived catecholaldehydes contribute to carbonyl stress and mitochondrial dysfunction in this tissue. While additional studies are necessary to completely understand the reactivity and cardiotoxicity of catecholaldehydes, these initial findings provide insight into a pathogenic mechanism by which MAO contributes to cardiac disease in diabetes (i.e. atrial fibrillation, cardiomyopathy). It is anticipated these results will inform future studies in experimental models concerning the role of MAO in cardiac diseases. Finally, our study suggests that targeting catecholamine metabolism and aldehyde detoxification in heart may have therapeutic benefits in patients with type 2 diabetes.

SUPPLEMENTAL DATA

Supplemental Table 1. Multiple reaction monitoring (MRM) parameters used for quantification of catecholamines

Analyte	Ionization Mode	m/z	MRM Transitions (Q1 mass>Q3 mass)	Collision Energy	Retention time (min)
DHBA	Positive	139.1	140.1>123.0 140.1>51.2	13	1.27
Dopamine	Positive	153.2	154.2>137.1 154.2>91.1	13	1.72
DOPAC	Negative	168.2	169.2>123.0 169.2>77.1	17	2.83
Norepinephrine	Positive	169.2	170.2>152.2 170.2>107.1	13	1.25
DOPAL	Negative	152.1	151.1>143.1 151.1>123.1	-17	1.96

Supplemental Table 2.1. MAO isoform activity (DA as substrate) and Risk for Diabetes

MAO-A Total (pmol/min/mg enzyme)			
	Diabetes	Non-Diabetes	P-value
Mean±SD	17723±9172	11603±3496	0.26†
			P_{trend}<0.051††
MAO-B Total (pmol/min/mg enzyme)			
Mean±SD	2582±8128	19281±8243	0.74
			P_{trend}<0.72††

Supplemental Table 2.2. MAO isoform activity (NE as substrate) and Risk for Diabetes

MAO-A Total (pmol/min/mg enzyme)			
	Diabetes	Non-Diabetes	p-value
Mean±SD	5943±3092	2673±1527	0.010†
			P_{trend}<0.0018††
MAO-B Total (pmol/min/mg enzyme)			
Mean±SD	6759±3000	4044±3228	P=0.010†
			P_{trend}<0.010††

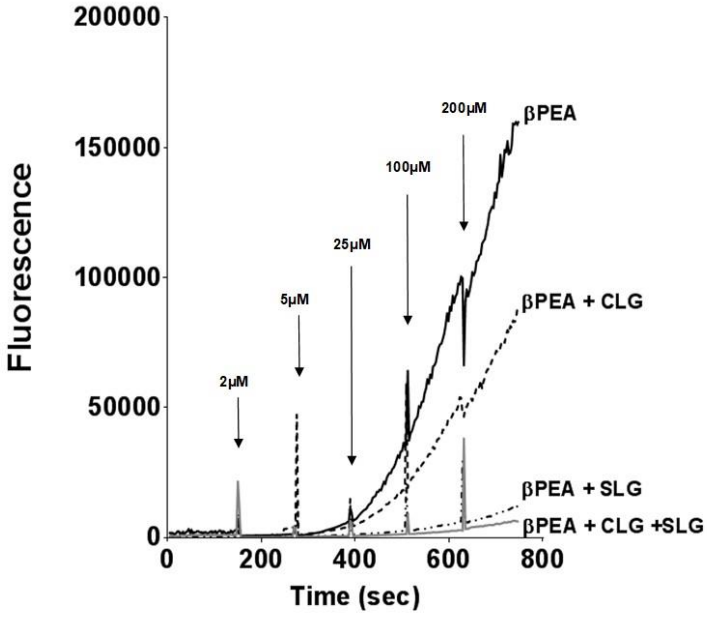
† p-value computed using Friedman's Nonparametric test for central tendency adjusting for age, sex and race.

†† p-value computed using the likelihood ratio trend test adjusting for age, sex and race.

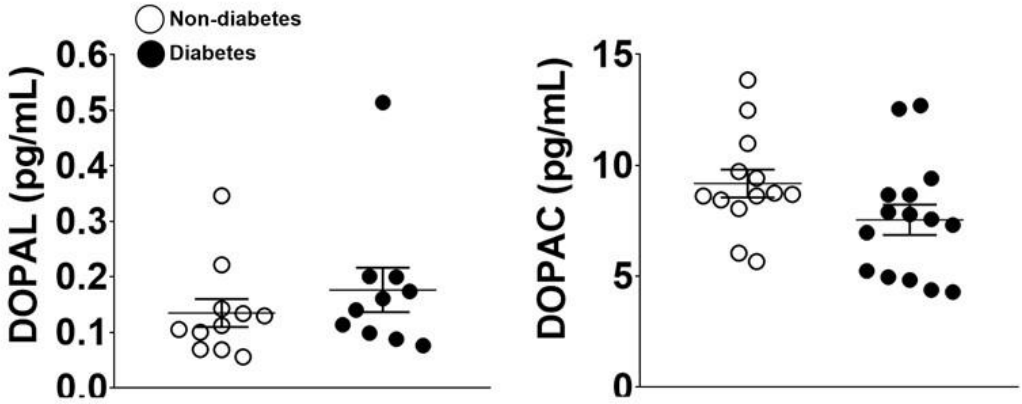
Supplemental Table 3. Fragmentation confirmation of tyrosine pathway metabolites.

Metabolite	Ion	m/z	Retention Time	Matched Reported Fragments
HPAA	$[M+H]^+$	153.1251	11.12	*153.125 (45%) *135.115 (13%) *121.026 (25%) *107.083 (4%) *95.046 (12%) *77.0363 (1%)
DHPG	$[M+H]^+$	171.0992	9.43	*171.145 (12%) *153.089 (6%) *135.113 (4%) *111.041 (61%) *93.070 (2%) *89.067 (3%) 83.083 (4%) *69.068 (6%) 55.053 (2%)
L-DOPA	$[M+H]^+$	199.1659	11.63	*199.177 (4%) 157.972 (20%) *111.114 (33%) 83.084 (4%) *71.0472 (2%) 69.067 (31%) 57.067 (2%) 55.052 (4%)

*Matched reported fragments for the metabolite. Hydroxyphenylacetic acid (HPAA), Dihydroxyphenylglycol (DHPG).


200000
150000
100000
50000
0
Fluorescence
0
200
400
600
800
Time (sec)
2μM
5μM
25μM
100μM
200μM
βPEA
βPEA + CLG
βPEA + SLG
βPEA + CLG +SLG

Supplemental Figure 1: Validation of cardiac MAO-A and –B isozyme activity assay. Representative fluorescent traces of continuous H_2O_2 production from MAO isozyme-specific activity in cardiac tissue lysate. Shown are experimental traces of total MAO activity supported by β-phenylethylamine (βPEA) (black solid line), MAO-B only (MAO-A is inhibited by Clorgyline (Clg), black dashed line), MAO-A only (MAO-B inhibited by Selegiline (Slg), gray dashed line), and background rate with both isozymes inhibited (gray solid line).


Non-diabetes
Diabetes
DOPAL (pg/mL)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
DOPAC (pg/mL)
15
10
5
0

Supplemental Figure 2: Concentrations of DOPAL and DOPAC within plasma. LC-MS analysis of DOPAL and DOPAC concentrations within plasma from patients with and without type 2 diabetes (n=13/group). Data are shown as ±SEM

CHAPTER 3:

BIOCHEMICAL CHARACTERIZATION OF THE CATECHOALDEHYDES REACTIVITY OF L-CARNOSINE AND ITS THERAPEUTIC POTENTIAL IN HUMAN MYOCARDIUM

From **Nelson MM**, Builta ZJ, Monroe TB, Doorn JA, Anderson EJ. Biochemical characterization of the catecholaldehyde reactivity of L-carnosine and its therapeutic potential in human myocardium. *Published Amino Acids. 2018 Sept 6.*

INTRODUCTION

Catabolism of norepinephrine (NE) and dopamine (DA) by monoamine oxidase (MAO) constitutes the principal route of neurotransmitter metabolism in oxidative tissues. MAO metabolizes norepinephrine (NE) and dopamine (DA) to produce the catecholaldehydes 3,4-dihydroxyphenylglycolaldehyde (DOPEGAL) and 3,4-dihydroxyphenylacetaldehyde (DOPAL), respectively, and H_2O_2 (43). Products of oxidative stress inactivate the enzymes responsible for catecholaldehyde metabolism, amplifying their levels and toxicity (69-71). Both DOPEGAL and DOPAL have been demonstrated to be far more cytotoxic and reactive than the parent catecholamines (i.e., NE and DA) or any other known metabolites (129). The surprisingly high toxicity appears to stem from the reactivity of both catechol and aldehyde groups. Due to their electrophilic nature, catecholaldehydes form covalent and very stable adducts with protein amines (e.g., Lys) and other biological molecules, permanently modifying their structure and function, as previously reported (61, 62, 66, 130).

Carnosine is an endogenous histidyl dipeptide found in millimolar concentrations in skeletal muscle, heart and brain (83). The dipeptide has therapeutic effects, exhibiting pH buffering, metal chelating, and antioxidant properties. Numerous groups have documented the ability of carnosine to detoxify and form stable conjugates with oxidized sugar- and lipid-derived aldehydes, such as acrolein and 4-hydroxy-2-nonenal (4HNE), yet the ability of carnosine to conjugate catecholaldehydes has, to our knowledge, never been examined (84, 88, 131-134). Therefore, the aim of this study was to investigate if carnosine can attenuate reactivity and toxicity of catecholaldehydes through the formation of catecholaldehyde-carnosine conjugates. In addition, we sought to compare reactivity of carnosine with other endogenous nucleophiles and antioxidants, such as the tripeptide glutathione (GSH), and L-Cys, to determine if carnosine is a novel scavenger of catecholaldehydes.

RESEARCH DESIGN AND METHODS

Chemicals. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise described. DOPAL was purchased from Cayman Chemicals or synthesized via the method of Fellman (135, 136). DOPEGAL was biosynthesized using commercially available MAO (137).

Reactivity of Biogenic Aldehydes. DOPAL (100 µM) was incubated with 1 mM carnosine, 1 mM GSH or 1 mM L-Cys at 37 ºC in 50 mM sodium phosphate buffer, pH 7.4. At time points (0, 30, 60, 90 and 120 min), aliquots were taken from the reaction

mixture and the reaction stopped by diluting 1:10 with 0.1% trifluoroacetic acid (TFA). Samples were stored at -20ºC in freezer or 4ºC in auto-sampler prior to analysis. The concentration of DOPAL was measured via a 1200 Series Agilent Capillary HPLC system with a photodiode array detector (202 and 280 nm). Separation was achieved using a Phenomenex C18 Luna column (1x150 mm) with flow rate of 50 µL/min and mobile phase of 0.1% TFA with 6% acetonitrile (isocratic). Dilution of the DOPAL-conjugates in 0.1% TFA and incubation for up to 2hrs at 37ºC did not result in appreciable decomposition of the DOPAL-carnosine conjugate. 4HNE (100 µM) was incubated with 1 mM GSH or 1 mM carnosine and time-dependent change in concentration of 4HNE was measured spectrophotometrically at 224 nm. The reaction of 100 µM 4HNE with 1 mM carnosine was allowed to equilibrate for 20 min before recording the time-dependent change in [4HNE] to be used in the rate calculation. Given the rapid reaction of 100 µM 4HNE with 1 mM GSH, the time-point at 60 min (>7 half-lives) was used as the blank for the reaction. Kinetics for the reaction of DOPAL and 4HNE with nucleophiles (e.g., carnosine, L-Cys) were pseudo-first order given the nucleophile (e.g., 1mM carnosine) was in 10-fold excess of the electrophile (e.g., 100 µM DOPAL). During the course of the reaction, the change in [carnosine] would be very little compared to the change in [DOPAL].

Human research participants, informed consent and collection of myocardial samples. This study was reviewed and approved by the Institutional Review Board of the Brody School of Medicine at East Carolina University. Enrolled in the study were patients undergoing elective cardiac surgery who were between the ages of 50 to 70

and presented without a prior history of cardiovascular surgery or arrhythmias. Informed consent for participation in the study was obtained by a member of the research team who was not involved in data collection or analysis. Prior to the initiation of cardiopulmonary bypass, a purse-string suture was made around the portion of the right atrial appendage (RAA) for insertion of the venous cannula. A section of the RAA was then excised and immediately rinsed in phosphate buffered saline. Myocardium was dissected from the endocardial side of the right atrial biopsy and placed in ice-cold Buffer X (7.23mM K_2EGTA, 2.77mM CaK_2EGTA, 20mM Imidazole, 0.5mM DTT, 20mM Taurine, 5.7mM ATP, 14.3mM PCr, 6.56mM $MgCl_2$-$6H_2O$, 50mM K-MES) and immediately processed for mitochondrial measurements. A small portion was frozen in liquid N_2 and maintained at -80°C for protein analysis.

Preparation of permeabilized myofiber and mitochondrial O_2 measurements

Techniques used for permeabilization of human cardiac myocardium have been previously described (31, 36). Briefly, myocardial samples were placed in ice-cold Buffer X containing 3mg/mL Collagenase Type I on a rocker at 4°C for 30 minutes. Myocardial tissue was cleared of connective tissue and separated into fibers along the longitudinal axis followed by permeabilization in Buffer X containing 50µg/mL saponin for 30 minutes at 4°C. The fibers were washed in Buffer Z (in mM: 105 K-MES, 30 KCl, 1 EGTA, 10 KH_2PO_4, 5 $MgCl_2$-$6H_2O$, 5mg/mL BSA) containing 20µM blebbistatin and placed on a rotator at 4°C until analysis (< 1 hour). Mitochondrial O_2 consumption was measured using the O_2K oxygraph system (Oroboros Instruments) at 30°C with continuous stirring. Permeabilized fiber bundles were placed in a chamber with Buffer Z Lite (in mM: 105 K-

MES, 30 KCl, 1 EGTA, 10 KH_2PO_4, 5 $MgCl_2$-$6H_2O$, 0.5mg/mL BSA, 0.05 palmitoyl-L-carnitine, 2 malate). State 3 respiration was initiated by the addition of 5 mM ADP and followed by titrations of DOPAL. A second fiber from each patient was pretreated with 10 mM carnosine, 1 mM GSH or 1 mM L-Cys for 5 minutes prior to measuring state 3 respiration.

Analysis of catechol-modified proteins in isolated mitochondria. Mitochondria from myocardial atrial samples were isolated with methods adapted from previous studies (138). In brief, fresh myocardium was minced on ice for 4 minutes and underwent a 2-minute trypsin digestion. To halt trypsin activity, trypsin inhibitor was added, and the mixture was placed in a 50mL conical tube and allowed to settle. The supernatant was removed, and the minced tissue was resuspended in mitochondrial isolation medium-MIM (in mM: 300 sucrose, 10 Na-HEPES, 0.2 EDTA) and homogenized using a pre-chilled Dounce homogenizer. The mixture was homogenized slowly with 10-12 strokes. The homogenate underwent differential centrifugation steps and the pellet of mitochondria were resuspended in MIM + BSA. Isolated mitochondria (50 µg) were incubated for 3 hours at 37°C with 75 µM NE and increasing concentrations of carnosine (1, 2.5, 5, 10, 25 mM). Mitochondrial proteins were subject to SDS-PAGE under reducing conditions followed by transfer to a nitrocellulose membrane. Nitro-blue tetrazolium (NBT) was used to detect catechol-modified protein adducts. NBT, a redox-cycling dye, stains nitrocellulose blue in the presence of catechol functional group (61, 112). The nitrocellulose membrane was incubated with 0.24 mM NBT in 2 M potassium

glycine buffer and rinsed in distilled water. The membrane was imaged using a ChemiDoc Imaging System (Bio-rad).

Statistical analysis. Data from this study are presented as means ± SEM or means ± SD, as noted. A student's paired t-test was used to define differences between ADP-stimulated respiration +/- carnosine within patient samples. Statistical significance was denoted at $p<0.05$ and statistical tests and linear regression were performed with GraphPad Prism (GraphPad Prism, La Jolla, CA).

RESULTS

Carnosine rapidly conjugates catecholaldehydes. Carnosine (1 mM) reacted with DOPAL (100 µM) and demonstrated pseudo-1st order kinetics with time-dependent loss of the catecholaldehyde in the presence of the dipeptide; however, much less reactivity was observed following incubation of carnosine (1 mM) with the lipid peroxidation product 4HNE (100 µM) (Figure 7A). The pseudo-1st order kinetics were anticipated given that [carnosine] >> [DOPAL], such that there was little change in [carnosine] with depletion of [DOPAL].

In contrast, GSH (1 mM) rapidly scavenged 4HNE (100 µM) with a half-life of approximately 3 min for 4HNE but exhibited no reactivity towards DOPAL (100 µM) over the course of 120 min (Figure 7B). Like carnosine, L-Cys (1 mM) exhibited scavenging activity towards DOPAL (100 µM); however, the kinetics may not be pseudo-1st order and there appeared to be reversibility for conjugate formation (Figure 7C). Work is in

progress to characterize the rapid kinetics of DOPEGAL reactivity towards carnosine and protein nucleophiles.

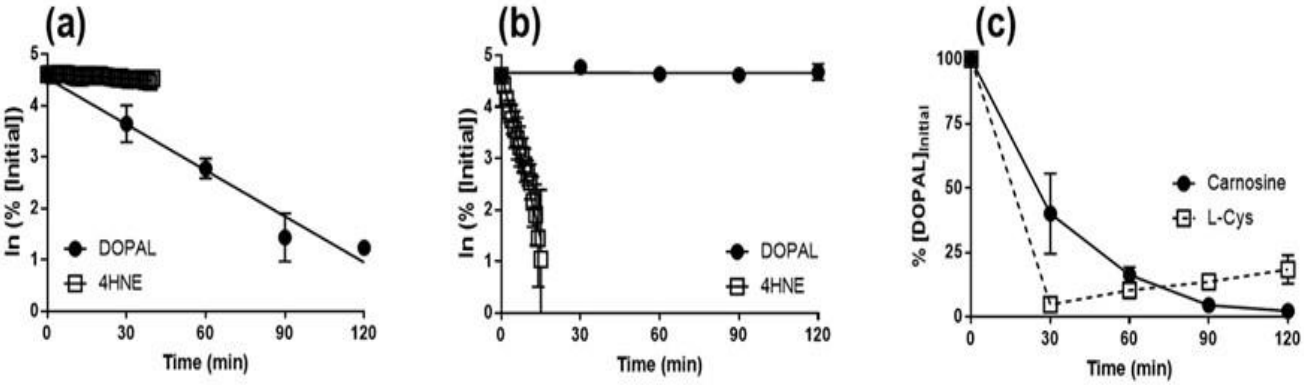

(a)
ln (% [Initial])
DOPAL
4HNE
Time (min)
(b)
ln (% [Initial])
DOPAL
4HNE
Time (min)
(c)
% [DOPAL]Initial
Carnosine
L-Cys
Time (min)

Figure 7. Comparison of DOPAL and 4HNE scavenging by antioxidants. The reaction of (a) 1 mM carnosine with 100 µM DOPAL or 100 µM 4HNE; (b) 1 mM GSH with 100 µM DOPAL or 100 µM 4HNE; (c) 100 µM DOPAL with 1 mM carnosine or 1 mM L-Cys. Data are reported as mean ± SD (n=3, carnosine with DOPAL or 4HNE; L-Cys with DOPAL), (n=4, GSH with 4HNE) or (n=2, GSH with DOPAL). Measured slopes for (a) and (b) were significantly non-zero ($p<0.01$) except for GSH with DOPAL ($p=0.97$).

Carnosine attenuates DOPAL toxicity in human cardiac mitochondria.

Permeabilized myofibers exposed to titrations of DOPAL revealed a dose-dependent decrease in state 3 respiration, even exhibiting a significant drop with the initial addition of 1μM DOPAL ($p<0.05$). Simultaneous incubation of DOPAL with 10 mM carnosine significantly attenuated the decrease in respiration across all concentrations of DOPAL (Figure 8A), while incubation with GSH and L-Cys under similar conditions did not mitigate the effect of DOPAL on respiration (Figure 8B, C).

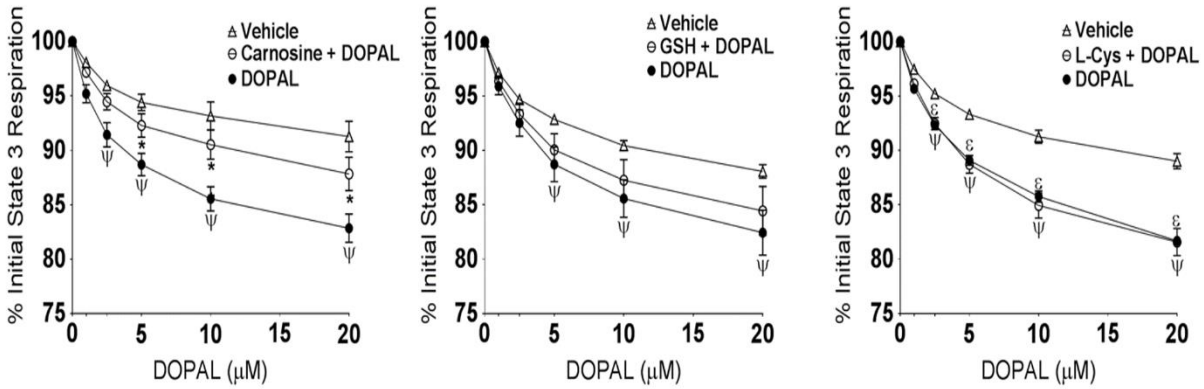

% Initial State 3 Respiration
100
95
90
85
80
75
0
5
10
15
20
DOPAL (μM)
Vehicle
Carnosine + DOPAL
DOPAL
GSH + DOPAL
L-Cys + DOPAL

Figure 8. Carnosine attenuates DOPAL-dependent decrease in state 3 respiration. Effect of DOPAL titration on state 3 respiration in permeabilized myofibers isolated from human RAA, either alone or following a pre-incubation with carnosine (**a**), GSH (**b**) and L-cysteine (**c**). Respiration was supported by palmitoyl-carnitine & malate. Data are reported as mean ± SEM, *p<0.05 vs. DOPAL, Ψ p<0.05 vs vehicle, **ε**p<0.05 L-Cys+DOPAL vs vehicle.

Carnosine mitigates catechol-protein modifications in human cardiac mitochondria. Isolated mitochondria incubated with NE showed a greater degree of NBT-staining (Figure 9) compared to the control lane (first lane), indicating increased presence of catechol-modified protein adducts. Pre-treatment of mitochondria with carnosine showed a concentration dependent decrease in catechol-modified adducts, with efficacy even at the lowest concentration of carnosine (2.5mM).

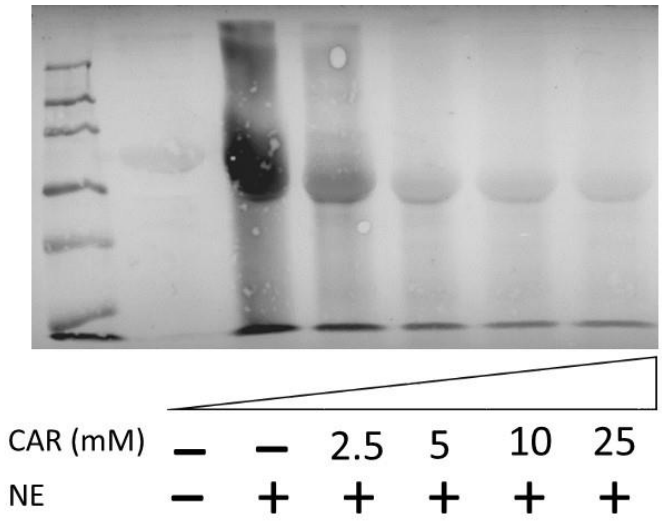

CAR (mM) – – 2.5 5 10 25
NE – + + + + +

Figure 9. Effect of carnosine on formation of catechol-modified adducts.

Representative blot showing catechol-adduct protein modifications in isolated human cardiac mitochondria when incubated with norepinephrine (NE) and/or increasing concentrations of carnosine.

DISCUSSION

The results from this study reveal, for the first time to our knowledge, the ability of carnosine to conjugate and sequester catecholaldehydes, the aldehyde byproduct of catecholamine deamination via MAO. Recent work has shown the rapid reactivity of DOPAL with protein amines, yielding stable adducts predicted to be a rearranged indole; however, the scavenging of DOPAL by carnosine was previously not known. Interestingly, GSH is not an effective scavenger of catecholaldehydes; however, GSH quickly reacts with the lipid peroxidation product 4HNE, while carnosine's reactivity is much slower (76). Such findings demonstrate the novel ability of carnosine, but not GSH, to scavenge a unique class of biogenic aldehydes (i.e., catecholaldehydes), and suggest a biological role for carnosine in cytoprotection against endogenous electrophiles formed via neurotransmitter metabolism. In addition, while L-Cys and carnosine readily reacted with DOPAL, carnosine but not L-Cys appeared to generate a stable conjugate that was not reversible during the reaction time-frame. It is known that L-Cys conjugates with aldehydes (e.g., acetaldehyde) to form thiazolidine products, and previous reports have noted this reaction to be reversible (139).

Our findings also indicate that carnosine mitigates catecholaldehyde-mediated disruptions in mitochondrial respiration, while GSH and L-Cys do not. Owing to the study design, our mitochondrial experiments cannot provide mechanistic insight as to how DOPAL decreases state 3 respiration in permeabilized myofibers, or perhaps more importantly, how carnosine is able to prevent the decrease in respiration. Never-the-less, the decrease in respiration observed in the presence of DOPAL signifies an interruption in enzymatic processes central to the efficiency of oxidative

phosphorylation. Therefore, additional studies are necessary to investigate which specific enzymes within the electron transport and/or phosphorylation systems are modified by catecholaldehydes, and the broad effect of these modifications.

Protein modification by catecholaldehyde adducts has been shown in work from our laboratory and others to cause toxicity in neuronal cell cultures and to contribute to the pathophysiology of neurological disease (49, 63, 130, 140, 141). Our finding that carnosine dose-dependently mitigates catecholaldehyde protein adduct formation following norepinephrine exposure in isolated human cardiac mitochondria indicates that 1) MAO content and activity is more than sufficient in human heart to generate catecholaldehydes when catecholamines are present; 2) there are numerous mitochondrial protein targets for these catecholaldehydes, as indicated by the large number of bands within each well of our NBT stain (Figure 9); and 3) carnosine is capable of blunting formation of catecholaldehyde protein adducts, even at physiologically relevant concentrations. In future studies, targeted proteomic- and metabolomic- based approaches will be necessary to elucidate the extent to which catecholaldehyde modifications alter cellular metabolism and mitochondrial energetics, and the potential for carnosine to mitigate these effects.

In summary, our results highlight the capacity of carnosine to conjugate and scavenge catecholaldehydes, thus expanding the repertoire of carnosine reactivity beyond those formed by lipid- and sugar-derived aldehydes. Such findings indicate carnosine to be a unique scavenger for catecholaldehydes, a property not seen for GSH. There are many clinical implications for these findings. The carnosine-DOPAL conjugate may be a unique biomarker for elevated levels of DOPAL and could be used

in a variety of clinical applications in patients at risk for neurological and cardiovascular diseases. Furthermore, these findings illustrate the therapeutic potential of carnosine in pathological states where increased MAO activity and sympathetic tone (i.e., catecholamine overload), and compromised aldehyde detoxification are known to be involved (142-144).

CHAPTER 4:

GENERAL DISCUSSION

Collectively the findings from Chapter 2 and 3 support the central hypothesis that MAO-derived byproducts disrupt mitochondrial bioenergetics and contribute to redox imbalance within myocardium from individuals with type 2 diabetes. Using MAO gene deletion or irreversible inhibitors, several studies eloquently demonstrated that MAO activity is involved in the development of left ventricular diastolic dysfunction often observed in diabetes and HF (52, 53, 55). Yet, these studies were not able to detail the mechanisms by which MAO byproducts led to the observed cellular changes. The results of our studies strongly suggest that it is through the ability of MAO-byproducts, specifically catecholaldehydes, to form catechol-protein conjugates and disrupt OxPHOS contributing to myocardial damage in type 2 diabetes. These findings link the mechanisms by which MAO activity may contribute to structural and functional alterations of LV observed within type 2 diabetes.

Within sympathetic cardiac nerve terminals, the release, uptake and storage of NE is maintained under tight regulation. Of the NE that is released at the presynaptic terminals, up to 90% is sequestered and returned by the norepinephrine transport (NET) while the remaining either enters circulation or is transported into myocardium and metabolized by MAO (145, 146). Several studies have shown increased sympathetic discharge and an inverse correlation between HbA1c and the expression of NET in myocardium from individuals with type 2 diabetes (147, 148). These results, in combination with findings from Chapter 2, suggest a disruption in the sympathetic

regulation of NE which may lead to greater myocardial uptake of NE within type 2 diabetes.

A significant finding from Chapter 2 is that MAO activity and expression are increased in individuals with type 2 diabetes. To our knowledge, this is the first report of alterations in cardiac MAO expression and activity within human subjects. Similar results have been reported using streptozotocin-induced diabetes that are more relevant to Type 1 Diabetes (55, 149-151). Although the mechanisms by which MAO expression and activity were increased were not ascertained within this dissertation, several studies indicate increased sympathetic signaling subsequent to hypoxia as a potential mechanism. In animal models, hypoxic/ischemic conditions contributed to increased MAO-A expression and activity and the expression and activities of both MAO isoforms were increased in left and right ventricles in patients with HF secondary to ischemic heart disease (57, 152, 153). The upregulation of MAO, especially MAO-A, due to hypoxia has been established, yet how hypoxia leads to the upregulation is still being explored. Several groups speculated that it is due to increase NE release during ischemic periods, but other groups have shown MAO-inhibition prior to hypoxia prevented the upregulation of MAO (154-156). The myocardium used in our studies was primarily from patients who suffered a myocardial infarction attributable to coronary artery disease, which indicates the myocardium was exposed to hypoxic conditions. Although hypoxic conditions due to coronary artery disease was a commonality between the metabolic groups, atherosclerotic lesions within coronary arteries in individuals with type 2 diabetes were more calcified, contained greater pro-inflammatory immune cells, and larger necrotic core size, suggesting increased rupture and greater

vascular smooth muscle death (157, 158). Furthermore, due to impairments in arterial vasodilation, abnormalities in coronary blood flow are observed in type 2 diabetes (159, 160). Based on these findings, coronary artery disease within type 2 diabetes could be more severe leading to greater global hypoxia compared to patients without diabetes and accounting for increased in MAO expression/activity. In addition, the myocardium used in this study was dissected from the right atrial appendage. Infarcted and ischemic areas most commonly occur within the smaller branches of the coronary arteries surrounding the ventricles, yet atrial infarctions comprise 17% of all infarctions and can occur concomitantly with ventricular infarctions (161, 162). Moreover, 98% of atrial infarctions occur within the right atrial appendage (161, 163). As ventricular tissue is difficult to obtain, these findings support using right atrial myocardium as a representative of myocardial damage within type 2 diabetes.

Results from Chapter 2 showed MAO-derived byproducts decreased ATP production in Pmfbs from individuals with type 2 diabetes. ATP synthase and adenine nucleotide translocase (ANT1) are two sites integral for ATP production via OxPHOS. ANT, located on the inner mitochondrial membrane, facilitates the exchange of ADP into the mitochondria and ATP into the cytosol therefore dictating the supply of ADP for OxPHOS (164). Post-translational modifications of ANT, including phosphorylation of tyrosine residues within the substrate binding site, have shown to decrease ADP exchanging activity (165). This mechanism was unlikely attributable for our observations as the phosphorylation of these residues provokes the opening of the mitochondrial permeability transition pore that would disrupt oxygen consumption, an effect that was not observed in our study (165). Several studies have shown 4HNE and acrolein

inhibited ANT activity in a concentration dependent manner through carbonyl modifications (166-168). Additionally, a recent study showed increased 4HNE protein adducts within myocardium from individuals with type 2 diabetes (30). Based upon the oxidative microenvironment observed in myocardium from individuals with type 2 diabetes, it is possible that ANT was previously modified by PUFA-derived aldehydes and the additional reactivity of MAO-derived byproducts overwhelmed the activity of ANT, leading to decreased exchange of ADP/ATP, and thus decreased ATP production.

The metabolomic results showed a propensity towards decreased catecholaldehyde detoxification in myocardium from individuals with type 2 diabetes. Although DOPAL and DOPEGAL could not be quantified due to the instrumentation used, catecholaldehyde production and reactivity are suggested to be higher in myocardium from individuals with type 2 diabetes based upon the observed increase in catechol-protein adduct formation and decreased ALDH2 activity. A primary focus of Chapter 2 was DOPAL and ALDH2 activity, however investigating DOPEGAL and AR activity are equally relevant. This is an apparent limitation of the study especially since NE is the primary catecholamine found within human heart (169, 170). Interestingly, AR activity has been suggested to be a pathogenic contributor to cardiac dysfunction observed in type 2 diabetes. The metabolism of reactive aldehydes to alcohols is a primary role of AR, yet in hyperglycemic conditions, it is involved with an aberrant pathway which metabolize excess glucose. Glycolysis is the central pathway for glucose metabolism, but after hexokinase becomes saturated, excess glucose enters the polyol pathway (171). In this pathway AR uses NADPH as a cofactor to convert glucose to sorbitol followed by its conversion to fructose (171). The increased rate of converting

glucose to sorbitol also increases the oxidation of NADPH. NADPH is required to reduce oxidized glutathione, a major antioxidant of the cell, yet due to the increased use of NADPH within the polyol pathway, the reduction of oxidized glutathione is attenuated leading to a redox imbalance (172). Within type 2 diabetes, AR activity is suggested to contribute to diastolic dysfunction, as studies which used AR inhibitors observed improvement in diastolic function and oxidative stress (173, 174). The dual ability of AR to detoxify reactive aldehydes (e.g. catecholaldehydes) while simultaneously driving redox imbalance by metabolizing glucose, reveals the complexities of oxidative stress and detoxification pathways within type 2 diabetes.

The results from Chapter 2 highlighted deficient ALDH2 activity in individuals with type 2 diabetes which segued into investigating a potential compound to enhance catecholaldehyde detoxification. Within our study carnosine was able to irreversibly bind DOPAL in a pseudo-first order kinetics manner, prevented DOPAL-dependent decrease in oxygen consumption within Pmfbs, and mitigated the formation of mitochondrial catechol-protein adducts. In these studies, carnosine also extended greater scavenging reactivity with DOPAL compared to GSH or L-Cys and showed a different scavenging profile with DOPAL compared to 4HNE. While our study showed carnosine to extend minimal reactivity towards 4HNE *in vitro*, numerous studies have shown that carnosine binds to 4HNE to form carnosine-4HNE conjugates *in vivo* (87, 93, 175). Endogenous levels of carnosine are reduced in muscle of type 2 diabetes, proposed by increased CN1 activity, which suggests decreased availability of carnosine to scavenge aldehydes (97, 176, 177). As carnosine is readily metabolized in plasma by CN1, several groups have investigated modifying the structure of carnosine to delay metabolism and prolong

reactivity. A recent study used oral carnosinol, a carnosine derivative which bypasses metabolism by CN1, that exhibited greater scavenging reactivity to 4HNE and acrolein compared to carnosine (94). These findings, in combination with findings from Chapter 3, provide evidence of the therapeutic potential of carnosine or carnosine analogues to improve redox balance and mitochondrial respiration in type 2 diabetes by attenuating reactivity of PUFA-derived aldehydes and MAO-derived byproducts.

FUTURE DIRECTIONS

In order to better understand the pathogenic role of MAO within cardiac dysfunction observed in type 2 diabetes, future studies are warranted and will attempt to answer the following:

1) *In this study myocardium from individuals with type 2 diabetes exhibited increased MAO activity, even after normalizing to enzyme content, which suggests an enhanced catalytic activity within the diabetic condition. Based upon this finding, how is MAO catalytic activity altered in type 2 diabetes?*
   Alterations in catalytic activity within disease states could be a result of pre- or post-translational modifications. Several studies have investigated polymorphisms of MAO-A within depression and correlated specific polymorphisms with decreased catalytic activity (178, 179). Based upon these studies, there does appear to be a causal link between disease state and genotype. These studies provide evidence to propose investigations of genotyping MAO-A within myocardium from individuals with and without type 2 diabetes. Combining the obtained results with published structural studies could

provide insight into potential substitutions within the substrate binding and active site. Additionally, to investigate post-translational modifications (PTM), chemical label and enrichment could be performed prior to proteomic analysis in order to detect most common PTM within specific amino acid residues. Further analysis of proteomic study would need to be performed to determine if specific modulations contribute to altered catalytic activity.

2) *Are MAO-derived byproducts synthesized in cardiomyocytes transmissible to and disrupt function of surrounding cells, including fibroblast, immune cells, endothelial cells, smooth muscle cells, adipocytes and other cardiomyocytes?*

The heart is comprised of multiple networks of cells of which cardiomyocytes only make up approximately 30%, therefore by understanding if MAO-derived byproducts are transmissible would provide insight into how localized myocardial damage caused by MAO-derived byproducts can affect the function of other cell types (14, 180). A recent study showed that DOPAL and DOPAC produced and released by PC-12 cells were transported into co-cultured neuronal cells which then led to cytotoxic effects (181). A similar initial study could investigate the release and uptake of MAO and ALDH2 derived byproducts within cardiomyocytes and cardiac fibroblasts. Media could be removed from cardiomyocytes exposed to catecholamines and run through HPLC to detect MAO-derived byproducts. If byproducts are present, fibroblasts could be exposed to cardiomyocyte-extracted media and measured for cellular alterations including fibrosis progression (TGF-β, collagen deposition, MMP signaling), cellular proliferation, and alterations in

phenotype. These findings could help to underscore the greater impact of MAO within cardiac dysfunction observed in type 2 diabetes.

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APPENDIX: IRB APPROVAL LETTER

EAST CAROLINA UNIVERSITY
University & Medical Center Institutional Review Board
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Notification of Continuing Review Approval: Expedited

From: Biomedical IRB
To: Jacques Robidoux
CC:

Date: 8/30/2018
Re: CR00007142
UMCIRB 09-0669
MYOCARDIAL REDOX STATUS, CATECHOLAMINE METABOLISM AND POST-OPERATIVE ARRHYTHMIA

The continuing review of your expedited study was approved. Approval of the study and any consent form(s) is for the period of 8/29/2018 to 8/28/2019. This research study is eligible for review under expedited category #9. The Chairperson (or designee) deemed this study no more than minimal risk.

Changes to this approved research may not be initiated without UMCIRB review except when necessary to eliminate an apparent immediate hazard to the participant. All unanticipated problems involving risks to participants and others must be promptly reported to the UMCIRB. The investigator must submit a continuing review/closure application to the UMCIRB prior to the date of study expiration. The Investigator must adhere to all reporting requirements for this study.

Approved consent documents with the IRB approval date stamped on the document should be used to consent participants (consent documents with the IRB approval date stamp are found under the Documents tab in the study workspace).

The approval includes the following items:

Document	Description
Amended protocol Sept 2014(0.02)	Study Protocol or Grant Application
Informed consent POAF JR 9-05-2017(0.01)	Consent Forms
Research Strategy for 1R01HL122863(0.01)	Study Protocol or Grant Application
Revised protocol for 09-0669 Sept2011.doc(0.01)	Study Protocol or Grant Application

The Chairperson (or designee) does not have a potential for conflict of interest on this study.

Study Staff Roles and Responsibilities

1.0 * Click on the UPDATE button beside each person's name to provide the responsibilities for each study staff member:

	Name	Role	Responsibilities
View	Jacques Robidoux	Principal Investigator	Enters data on paper research records, Data management, Collects data/specimens, Communicates with IRB, Trains research team members
View	Andy Kiser	Sub-Investigator	Data management, Educates participants, families, or staff, Performs observations
View	Ethan Anderson	Sub-Investigator	Enters data on paper research records, Data management, Communicates with IRB
View	Jimmy Efird	Sub-Investigator	Data management
View	Patricia Crane	Sub-Investigator	Performs observations, Trains research team members, Dr. Patricia Crane will be providing us with critical expertise regarding cardiovascular variables such as ECG, heart rate, etc, in the patients enrolled in our study.,
View	Ashton Hathaway	Other Study Staff	Screens potential participants, Obtains Informed Consent, Data management, Collects data/specimens
View	Margaret Ann Nelson	Other Study Staff	Obtains Informed Consent, Data management, Collects data/specimens
View	Amanda Lucas	Other Study Staff	Performs observations
View	Divya Madipally	Other Study Staff	Obtains Informed Consent, Data management, Collects data/specimens
View	Lisandra de Castro Brás	Other Study Staff	Data management, Collects data/specimens